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<p>(54) Title: PARAPOXVIRUS VECTORS</p> <p>(57) Abstract</p> <p>The invention is directed to parapoxvirus vectors. Specifically provided are orf virus vectors containing exogenous DNA. The exogenous DNA may encode a heterologous peptide or polypeptide of which expression is desired, or may encode an antigen capable of inducing an immune response. The capacity to express antigens make these vectors suitable for use in vaccines.</p>			

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PARAPOXVIRUS VECTORS

TECHNICAL FIELD

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This invention relates to parapoxvirus vectors, methods for their construction, and uses thereof.

BACKGROUND OF THE INVENTION

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Poxviruses are large DNA viruses which replicate within the cytoplasm of infected cells. A number of members of the poxvirus family have been used to express foreign genes. These members include vaccinia virus and avipox virus. Such viruses have the potential to deliver vaccine antigens to a variety of animal species. However, the use of modified 15 vaccinia virus and avipox viruses are subject to a number of drawbacks.

Vaccinia virus has a wide host range in mammals. Accordingly, there is a significant risk of cross-species infection and consequent spread of disease from one species to another. This represents a significant disadvantage for any vector being used in the environment.

20

A further disadvantage is that vaccinia virus especially, has been shown to cause a febrile response and scarring in humans and occasionally, serious disease in an infected animal.

Avipoxviruses are more variable in their host range specificity, and while they will not generally propagate in mammals, they will often undergo an abortive infection sufficient to induce an immune response to at least some foreign genes if they are incorporated into the genome of the avipoxvirus and are expressed under control of the appropriate promoter.

30 Also the first infection with a vaccinia virus vector will induce an immunity to the vector such that it may limit the potential of a subsequent infection with the vector to deliver a full dose of antigen.

In the agricultural context, a major limitation to livestock production is the control of 35 parasitic diseases. As drench resistance builds up in farmed animal populations, and consumer resistance to the use of chemical agents in livestock production also increases, there is a need for alternative means of disease control. Use of cheap, safe and effective

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vaccines using parapox virus vectors to deliver antigens to the host is one alternative solution which addresses these problems.

The concept of parapox virus vectors and more particularly orf virus vectors is disclosed generally by Robinson, A.J. and Lytle, D.J. "*Parapoxviruses: their biology and potential as recombinant vaccines*" in Recombinant Poxviruses, Chapter 9, 306-317 eds M.Binns and G. Smith CRC Press, (1992), Boca Raton. However, there is no teaching in the reference of suitable gene insertion sites or sequences coding therefor which would allow orf virus to be used as a vector.

It is therefore an object of the present invention to provide a virus vector which goes some way toward overcoming the disadvantages outlined above in relation to existing poxvirus vectors or which at least provides the public with a useful choice.

15 SUMMARY OF THE INVENTION

Accordingly, in one aspect, the present invention provides a parapoxvirus vector comprising a parapox virus containing exogenous DNA.

20 Preferably, the parapox virus is orf virus.

Desirably, the exogenous DNA encodes at least one gene product, and most usefully this product will be an antigen capable of inducing an immune response.

25 In addition, the exogenous DNA preferably further encodes at least one gene product which is a biological effector molecule, most usefully a cytokine which is capable of acting as an immunological adjuvant.

In addition, the exogenous DNA also preferably encodes a peptide moiety expressed as 30 a hybrid or chimeric protein with a native virus protein.

Also within the scope of the invention are fragments or variants of the vector having equivalent immunological activity.

35 It is desirable that the exogenous DNA be incorporated in a non-essential region of the virus genome.

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The exogenous DNA is preferably under the control of a poxvirus promoter, and conveniently an orf virus promoter.

In a further aspect, the present invention provides a method for the production of 5 parapoxvirus vectors, replicable transfer vectors for use in the method of the invention and hosts transformed with these vectors.

In a further aspect the invention consists in a vaccine which includes a parapoxvirus vector defined above in combination with a pharmaceutically acceptable carrier and 10 optionally or alternatively, an adjuvant therefor.

In a still further aspect the present invention relates to the use of parapoxvirus vectors to prepare heterologous polypeptides in eukaryotic cells comprising infecting cells with the parapoxvirus vector and isolating the heterologous polypeptide once expressed.

15 Although the invention is broadly as described above, it will be appreciated by those persons skilled in the art that the invention is not limited to the foregoing but also includes embodiments of which the following gives examples. In particular, certain aspects of the invention will be more clearly understood by having reference to the accompanying 20 drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents a map of the genomes of the orf virus strains NZ-2, NZ-7 and NZ-10 25 showing cleavage sites for the restriction endonuclease *KpnI*. The genomes are double stranded DNA molecules and are represented as horizontal lines. The positions of the endonuclease cleavage sites on each genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet.

30 Figure 2 represents a nucleotide sequence of a region of the *KpnI E* fragment of the orf virus strain NZ-2 genome. The sequence underlined with a dashed line contains potential insertion sites. The sequence underlined with colons represents that portion of a vascular endothelial growth factor like gene that contains potential insertion sites.

35 Figure 3 represents a nucleotide sequence of a region of the *KpnI D* fragment of the orf virus strain NZ-7 genome in Figure 1. The sequences underlined with a dashed line represent sites for the insertion of foreign genes. The sequence underlined with colons

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represents that portion of a vascular endothelial growth factor-like gene that contains potential insertion sites.

Figure 4 represents a map of the genome of the orf virus strain NZ-2 showing cleavage sites for the restriction endonuclease *Hind*III. The genome is a double stranded DNA molecule and is here represented as a horizontal line. The positions of the endonuclease cleavage sites on the genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet. The region comprising part of fragment F, all of fragments J and I and part of fragment E for which the DNA sequence has been determined is shown. Open reading frames encoding putative genes are shown. The open reading frames encoding the putative genes (H)I1L and (H)I2L contain potential insertion sites. In addition the intergenic regions between *rpo*132 and (H)I1L, (H)I1L and (H)I2L, (H)I2L and (H)E1L and (H)E1L and (H)E2L represent potential insertion sites.

Figure 5 represents the nucleotide sequence of the open reading frames depicted in Figure 4. The genes (H)I1L, and (H)I2L which contain potential insertion sites are underlined with colons. Potential insertion sites within intergenic regions are underlined with a dotted line. Putative promoter sequences are marked by asterisks.

Figure 6 represents a map of the genome of the orf virus strain NZ-2 showing cleavage sites for the restriction endonuclease *Bam*HI. The genome is a double stranded DNA molecule and is here represented as a horizontal line. The positions of the endonuclease cleavage sites on the genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet. The region comprising fragment *Bam*HI F and part of *Bam*HI C for which the DNA sequence has been determined is shown. Open reading frames encoding DNA topoisomerase (F4R) and the putative genes F1L, F2L, F3R and C1L are shown as unfilled arrows.

Figure 7 represents a nucleotide sequence of the *Bam*HI F fragment and part of the *Bam*HI C fragment of the orf virus strain NZ-2 genome shown in Figure 6. The sequences underlined with a dashed line represent potential insertion sites. The putative promoter sequences PF1L, PF2L, PF3R, PF4R and PC1R are marked by asterisks.

Figure 8 represents a map of the genome of orf virus strain NZ-2 showing cleavage sites for the restriction endonuclease *Bam*HI. The genome is a double stranded DNA molecule and is here represented as a horizontal line. The positions of the endonuclease cleavage

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sites on the genome relative to the ends of the genome are represented by vertical lines. Individual genome fragments that would be generated by digestion with the endonuclease are designated with letters of the alphabet. The region comprising fragments *Bam*HI H, *Bam*HI E, *Bam*HI G and part of *Bam*HI B for which the DNA sequence has been 5 determined is shown. Open reading frames encoding putative genes are shown as unfilled arrows. The position of a 3.3 kilobase pair deletion encompassing open reading frames E2L, E3L and G1L is shown.

Figure 9 represents a nucleotide sequence of a region of the *Bam*HI E fragment and 10 *Bam*HI G fragment of the orf virus strain NZ-2 genome shown in Figure 8. Potential insertion sites underlined by colons are present in the region which encodes for the putative genes E2L, E3L and G1L. Potential insertion sites within intergenic regions are underlined with a dotted line. Putative promoter sequences are marked by asterisks. The region located between the ITR junction and the marked endpoint of deletion is absent in 15 a variant strain derived from NZ-2.

Figure 10 represents nucleotide sequences from the orf virus genome strain NZ-2 that act 20 as transcriptional promoters. Early and late promoter sequences are indicated. For each sequence the left hand end is the 5' end.

Figure 11 is a diagram representing the steps in the construction of the plasmid pSP-PFlac.

Figure 12 is a diagram representing the steps in the construction of the plasmid pSP-SFPgpt32.

Figure 13 is a diagram representing the steps in the construction of the plasmid pFS-gpt.

Figure 14 is a diagram representing the steps in the construction of the plasmids pVU-DL104 and pVU-DL106.

Figure 15 is a diagram representing the steps in the construction of the plasmids ptov2 and 30 ptov3.

Figure 16 is a diagram representing the steps in the construction of the plasmid ptov6.

Figure 17 is a diagram representing the steps in the construction of the plasmid ptov8.

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Figure 18 is a diagram representing the steps in the construction of the plasmids pVU-DL45W and pVU-DL45WI.

5 Figure 19 is a diagram representing the steps in the construction of the plasmids pVU-DL45WIac and pVU-DL45WIac.

Figure 20 outlines a strategy for the generation of recombinant orf virus.

10 Figure 21A provides the nucleic acid sequence for the primers zxs-1, zxs-2, zxs-3 and zxs-4 used for the amplification of orf virus sequences used to create the transfer vector pTvec50.

15 Figure 21B provides the nucleic acid sequence for the modified intergenic region between the RNA polymerase subunit gene, rpo 132, and (H)I1L in pTvec50, showing new created restriction sites for the restriction enzymes *Apol*, *NsiI*, *NcoI* and *EcoRI*. The priming sites on the original OV sequence for the zxs-3 primer are marked by asterisks, the newly created transcriptional termination signal (TTTTTAT) is shown in bold type.

20 Figure 22 is a diagram representing the steps in the construction of the plasmids pTvec1 and pTvec-50.

Figure 23 is a diagram representing the steps in the construction of the transfer vectors pTvec50lac-1 and pTvec50lac-2.

25 In a first aspect the present invention provides a parapoxvirus vector comprising a parapox virus containing exogenous DNA. Preferably, the parapoxvirus is an orf virus. Orf virus has a relatively narrow host range being generally confined to sheep, goats, monkeys and man. The narrow host range avoids the disadvantage associated with the use of vaccinia virus as a vector in the environment. In particular, cross-species infection will be limited.
30 Most animals and birds would simply undergo an abortive infection of the orf virus, but the orf virus may still be capable of delivering an immunising dose of some antigens.

Accordingly, the narrow host range may allow the use of orf virus in animals normally resistant to infection with orf virus to stimulate an immune response. The orf virus may 35 also be particularly useful in delivering antigens to birds, where the virus does not propagate in avian species.

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Orf virus also has the advantage of being less virulent than vaccinia virus in man. Unlike vaccinia virus, orf virus does not cause a febrile response and lesions are shown to heal without scarring. Ideally the orf virus vector will lack its original virulence factor. Orf virus is reviewed in Robinson, A.J. and Balassu, T.C. (1981) *Contagious pustular dermatitis (orf)*. Vet Bull 51 771-761 and Robinson, A.J. and Lyttle, D.J. (1992) "Parapoxviruses: their biology and potential as recombinant vaccines" in Recombinant Poxviruses, Chapter 9, 306-317 eds M. Binns and G. Smith CRC Press, (1992), Boca Raton.

10 The term "containing exogenous DNA" as used herein refers to exogenous DNA which is incorporated into the virus genome.

Preferably, the exogenous DNA in the orf virus vector is a gene encoding a gene product or products. The gene product may be a heterologous peptide or polypeptide but most 15 usefully, the gene product is an antigen or antigens capable of eliciting an immune response in an infected host. Exogenous DNA encoding genes for a combination of antigens is also possible. The antigen(s) may also be treated with suitable inhibitors, modifiers, crosslinkers and/or denaturants to enhance its stability or immunogenicity if required.

20 Some examples of foreign genes of medical and veterinary importance which may potentially be incorporated into orf virus include HIV envelope protein, herpes simplex virus glycoprotein, *Taenia ovis* antigens, *Echinococcus granulosus* (hydatids) antigens, *Trichostrongylus* and antigens of gastrointestinal parasites such as *Haemonchus* and 25 *Ostertagia* or combinations thereof, but are not limited thereto.

Preferred antigens include *Taenia ovis* 45W, 16kd and 18kd antigens as disclosed in WO 94/22913 incorporated herein by reference.

30 In a further preferred embodiment, the exogenous DNA may further comprise a cytokine gene or genes coding for other biological effector molecules which modify or augment an immune response, in combination with the exogenous antigenic DNA. Preferred cytokine genes include γ interferon and the interleukins comprising IL-1, IL-2, IL-1 β , IL-4, IL-5, IL-6, IL-12 and most preferably IL-1, IL-2 and IL-12 either alone or in combination.

35 In another embodiment the exogenous DNA may further comprise one or more reporter genes and/or at least one gene coding for a selectable marker.

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Examples of suitable well known reporter genes include *Escherichia coli* β -galactosidase (*lacZ*), *Photinus pyralis* firefly luciferase (*lux*), secreted placental alkaline phosphatase (*SEAP*) and *Aequorea victoria* green fluorescent protein (*gfp*).

5 Selectable marker genes known and suitable for use in the present invention include xanthine-guanine phosphoribosyl transferase gene (*xgpt*), and neomycin phosphotransferase (*aphII*)

In a particularly preferred embodiment the exogenous DNA will comprise genes encoding 10 multiple antigens in combination with one or more biological effector DNA molecules to enhance immune response. In practical terms where multiple antigens are coded for they will generally number 20 or less, preferably 10 or less.

15 Additionally, the DNA preferably encodes a peptide moiety expressed as a hybrid or chimeric protein with a native virus protein.

In this embodiment of the invention the exogenous DNA encodes for a peptide sequence 20 that forms part of a virus protein. The native protein would retain its original properties but would exhibit additional antigenic epitopes, enzymatic properties or receptor-binding functions encoded by the exogenous DNA. Such a chimeric protein could be secreted, or could form part of the virus envelope or could form part of the virus capsid.

Also within the scope of the invention are fragments or variants of a vector of the 25 invention having equivalent immunological activity. Such variants may be produced by the insertion, deletion or substitution of one or more amino acids using techniques known in the art (Sambrook, J. Fritsch, E.F. and Maniatis, T. *Molecular Cloning, A Laboratory Manual* (Second Edition) Cold Spring Harbour Laboratory Press 1989).

As will be appreciated by the reader, it is also desirable for the foreign gene to be 30 incorporated into a non-essential region of the orf virus genome. In particular, the gene must be inserted into a region where it does not disrupt viral replication.

Surprisingly, the non-essential thymidine kinase gene, which is used as an insertion site 35 in vaccinia virus has not been found in orf virus. It was therefore necessary to identify alternative non-essential sites in orf virus.

Non-essential sites were identified following restriction enzyme mapping of orf virus DNA. DNA maps for orf virus strains NZ-2, NZ-7 and NZ-10 are shown in accompanying Figure 1.

5 Potential insertion sites are contained within restriction fragments *Kpn*I E of strain NZ-2, *Kpn*I D of strain NZ-7 and *Kpn*I D of strain NZ-10. Potential insertion sites are located in the restriction fragments *Bam*HI E and *Bam*HI G of strain NZ-2 shown in Figures 8 and 9. Other potential insertion sites have been identified as intergenic regions lying between regions encoding viral genes. Further examples are illustrated in Figures 4 and 5
10 10 (restriction fragments *Hind*III F, J, I and E of strain NZ-2) and in Figures 6 and 7 (restriction fragments *Bam*HI F and C of strain NZ-2). Other insertion sites are also within the scope of the invention, for example, any non-essential gene or intergenic region within the orf virus genomic DNA sequence. Moreover, one or more insertion sites may be selected and used at a time.

15 There are two currently preferred insertion sites. The first of these sites is the intergenic region between RNA polymerase subunit gene, *rpo*132 and the open reading frame of the presumptive gene (H) I1L (Figure 4). As shown in Figure 5 this insertion site is 90 nucleotides in length, extending from positions 11 to 96.

20 The second of the preferred insertion sites is the *Nco*I site located at the beginning of gene E3L (Figure 8). As shown in Figure 9 this insertion site is 61 nucleotides in length, extending from positions 2226 to 2286.

25 As will also be appreciated, if expression of the foreign gene is to be achieved, it must be under the control of a transcriptional promoter capable of expressing that gene.

A description of poxvirus promoters can be found in Moss, B. (1990). Regulation of vaccinia virus transcription. *Annu Rev Biochem.* 59, 661-688 incorporated herein by reference. As has been shown, poxvirus RNA polymerase complexes responsible for copying the gene to make a mRNA, will transcribe any gene that is preceded by a poxvirus promoter.

35 Preferably therefore, the promoter used will be a poxvirus promoter, and particularly a parapoxvirus promoter. The presently preferred promoter is an orf virus promoter. The orf virus promoter may be an early, intermediate or late promoter. Nucleotide sequencing has allowed the identification of a number of orf virus transcriptional promoters including

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early, intermediate and late promoters. Orf virus early and late promoters are shown in Figure 10.

One preferred orf virus promoter is the early promoter of the putative gene E1L originally described as ORF-3 by Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. *Virology*. 176, 379-389 and Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia virus-like early transcriptional control sequences flank an early gene in the orf parapoxvirus. *Gene*. 97, 207-212.

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Of the late promoters PF1L and PF3R are preferred. Initial studies on the relative strengths and the temporal expression of the promoters indicate that PF3R is an early-late promoter and is therefore the presently preferred promoter for expressing cloned genes encoding antigenic polypeptides. PF1L is a strong late promoter and is the presently preferred promoter for the expression of the β -galactosidase reporter gene. The orientation of the promoter and the gene it controls may be arranged as appropriate. Combinations of promoters may also be employed.

In a further aspect the invention consists in replicable transfer vectors suitable for use in preparing the modified orf virus vector of the invention. Replicable transfer vectors may be constructed according to techniques well known in the art (Sambrook, J, Fritsch, E. F. and Maniatis, T. *Molecular Cloning, A Laboratory Manual* (Second Edition) Cold Spring Harbour Laboratory Press 1989), or may be selected from cloning vectors available in the art.

25

The cloning vector may be selected according to the host cell to be used. Useful vectors will generally have the following characteristics:

- (i) the ability to self-replicate;
- (ii) the possession of a single target for any particular restriction endonuclease; and
- (iii) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

30 Two major types of vector possessing the aforementioned characteristics are plasmids and bacterial viruses (bacteriophages or phages). Plasmid vectors are preferred for use in the present invention. The plasmid vector will comprise a non-essential region of the orf virus genome, a foreign gene or genes under the control of one or more orf virus

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promoters, and a segment of bacterial plasmid DNA. The vector may be a linear DNA molecule but is preferably circular.

In the construction of a modified orf virus it is also an advantage to be able to distinguish
5 the modified virus from the unmodified virus by a convenient and rapid assay. Such assays include measurable colour changes, antibiotic resistance and the like. For rapid assay purposes, the virus vector desirably further includes at least one reporter gene such as *lacZ*, and and/or at least one selectable marker gene such as *x-gpt*.

10 In a preferred embodiment, the xanthine-guanine phosphoribosyltransferase gene (*x-gpt*) and the β -galactosidase gene are inserted into the plasmid vector under the control of suitable orf virus transcriptional promoters. The orientation of the inserted genes may also be important in determining whether recombinants can be recovered from transfections. Figure 14 shows the *x-gpt* gene in different orientations in pVU-DL101 and
15 pVU-DL102.

In a further aspect, the present invention provides a method for producing a modified orf virus vector. The method comprises transfecting the plasmid cloning vectors defined above into a selected host cell infected with orf virus. Suitable transfection techniques
20 are well known in the art, for example, calcium phosphate-mediated transfection as described by Graham, F. L. and Van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus type 5 DNA. *Virology*. 52, 456-467. Other techniques include electroporation, microinjection, or liposome or spheroplast mediated transfer but are not limited thereto. Preferably, liposome-mediated transfection is used. This method
25 is described by Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA*. 84, 7413-7417.

30 Upon transformation of the selected host with the cloning vector, recombinant or modified orf virus vectors may be produced. The modified virus may be detected by rapid assays as indicated above. For the preferred vectors the presence of the β -galactoside gene is detectable where clones give a blue phenotype on X-gal plates facilitating selection. Once selected, the vectors may be isolated from culture using routine procedures such as freeze-
35 thaw extraction. Purification is effected as necessary using conventional techniques. A strategy for the generation of modified orf virus is shown in Figure 20.

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The transformed host cells also form part of the invention. Many host cells are known in the art including bacterial, insect, plant and animal cells. Preferably, the host cell is a eukaryotic cell. Mammalian host cells are particularly desirable. The preferred host cells of the present invention are primary bovine testis cells or primary ovine testis cells (lamb testis cells).

As will be appreciated, in a further aspect of the invention, the protocol described above may be used to prepare heterologous polypeptides as well as antigens.

- 10 In another aspect, the present invention comprises a vaccine preparation comprising the modified orf virus which contains exogenous antigenic DNA, or a fragment or variant thereof having equivalent immunological activity thereto in combination with a pharmaceutically acceptable diluent or carrier and optionally or alternatively an adjuvant. Examples of suitable adjuvants known to those skilled in the art include saponins,
- 15 Freund's adjuvants, water-in-oil emulsions, glycerol, sorbitol, dextran and many others. Generally, adjuvants will only be used with non-living viral vaccine preparations.

In a further aspect, the present invention comprises a vaccine preparation comprising the modified orf virus which contains exogenous antigenic DNA in combination with

- 20 exogenous DNA encoding cytokine genes or genes for other biological effector molecules which may modify or augment an existing immune response.

The vaccine may be formulated in any convenient physiologically acceptable form. Vaccine preparation techniques for smallpox are disclosed in Kaplan, *Br. Med Bull.* 25, 25 131-135 (1969).

Most usefully, the vaccine is formulated for parenteral administration. The term "parenteral" as used herein refers to intravenous, intramuscular, intradermal and subcutaneous injection.

- 30 In addition the vaccine may be formulated for oral administration.

Other therapeutic agents may also be used in combination with the vaccine.

- 35 Where necessary, the vaccine may be administered several times over a defined period to maximise the antibody response to the foreign antigen.

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Other methods for inserting foreign genes into orf virus are also contemplated. Potentially, a restriction endonuclease that cuts orf virus DNA once may be used. The cleaved site may be removed following *in vitro* mutagenesis followed by joining by ligation. If the site is in an essential gene the mutagenesis may be arranged such that the 5 gene function is not affected. This is possible by substituting a base in a codon that lies wholly or partly in the restriction endonuclease cleavage site with another base that allows the new codon to code for the same amino acid but for that substitution to remove the cleavage site for that particular restriction endonuclease. The cleavage site could then be created within any non-essential gene by mutagenesis. This cleavage site then acts as a 10 site for the insertion of foreign genes. The insertion of foreign genes may be done outside the cell by removing the phosphate from the cleaved ends of the DNA to prevent recreation of uninterrupted orf virus DNA, joining a foreign gene which has phosphorylated ends into the orf virus DNA in a ligation reaction and then transfecting the resulting ligation mixture into cells permissive for orf virus. To recover the virus the 15 cell is infected with a poxvirus that was non-permissive for those cells, for instance fowlpox virus and primary bovine testis cells.

Non-limiting examples will now be provided.

20 Example 1 - Selection of a Suitable Cell Culture System

The source of cells for culture in the methods described in this application was calves of between one day and three months of age. The testicles were removed from the scrotum of the animal without anaesthetic by a veterinarian skilled in this procedure. The testicles 25 were removed with the tunica parietalis intact to keep the culture cells sterile. The tissue was transported on ice to the laboratory, and the testicular tissue removed from the testis, dispersed into single cells and small aggregates of cells and incubated in suitable culture vessels in culture medium by sterile procedures familiar to those skilled in the art.

30 Example 2 - Identification of Insertion Sites

The DNAs of various orf virus isolates have been physically mapped using restriction endonucleases. Such mapping has revealed that there are many different strains of the virus that can be distinguished by the size and order of the restriction endonuclease-generated fragments although strains may not necessarily differ in their phenotype. From 35 this data it was noted that there was a difference in size between two strains in a restriction endonuclease *Kpn*I fragment mapping to the right end of the genome (Robinson A.J., Barns, G., Fraser, K., Carpenter, E. and Mercer, A.A. (1987). Conservation and

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variation in orf virus genomes. *Virology*. 157, 13-23). These two strains were designated NZ-2 and NZ-7 and the fragments *KpnI E* and *KpnI D* respectively. NZ-7 contained the larger of the two fragments. The difference in size was about 1 kilobase pair. Another strain designated NZ-10 was seen to have a fragment, fragment *KpnI D* intermediate in
5 size between the corresponding fragments in NZ-2 and NZ-7 but located in the same relative position in the genome (see Fig. 1). This variability suggested that all or part of the region was non-essential and that within this fragment, a site in which to insert foreign DNA might be found. The regions described have subsequently been sequenced and potential insertion sites identified (Fig. 2 and Fig. 3).

10

Another potential insertion site was identified when DNA/DNA hybridization between strains, for example between NZ-2 and NZ-7, detected a region of non-homology extending over 2.75 kilobase pairs and this was mapped to a region about 30 kilobase pairs from the right end of the genome (Robinson A. J., Barns, G., Fraser, K., Carpenter,
15 E. and Mercer, A. A. (1987). Conservation and variation in orf virus genomes. *Virology*. 157, 13-23 and Naase, M., Nicholson, B. H., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). An orf virus sequence showing homology to the fusion protein gene of vaccinia virus. *J. Gen Virol.* 72, 1177 -1181) (Fig. 4). This region was then completely sequenced and two genes, HI1L and HI2L identified, each of which contains potential
20 insertion sites (Fig. 5).

A third potential insertion site was located in the centre of the genome where a size difference of 100 base pairs was seen between the *BamHI G* fragment in a strain designated NZ-41 and equivalent region in the other strains examined (Robinson, A. J.,
25 Barns, G., Fraser, K., Carpenter, E. and Mercer, A. A. (1987). Conservation and variation in orf virus genomes. *Virology*. 157, 13-23). The nucleotide sequence of the equivalent region in the genome of strain NZ-2, the *BamHI F* fragment, has been determined and two potential insertion sites identified (Fig. 6 and Fig. 7).

30 Fourthly, a spontaneous re-arrangement of the orf virus genome of strain NZ-2 was detected following serial propagation of the virus in cell culture. This re-arrangement resulted in the addition of 16 kilobase pairs of right-end DNA sequences to the left end and the deletion of 3.3 kilobase pairs of DNA from the left end. Genomic analysis of a transposition-deletion variant of orf virus reveals a 3.3 kbp region of non-essential DNA
35 (Fleming, S. B., Lyttle, D. J., Sullivan, J. T., Mercer, A. A. and Robinson, A. J. (1995). *J Gen Virol.*, 76, 2969-2978). The order of nucleotides making up the region of the genome that can tolerate a deletion has been deduced by the method of Sanger and three genes contained therein identified. These genes correspond to E2L, formerly ORF-1

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(Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. *Virology*. 176, 379-389), E3L formerly ORF-PP (Mercer, A. A., Fraser, K., Stockwell, P. A. and Robinson, A. J. (1989). A homologue of retroviral pseudoproteases in the parapoxvirus, 5 orf virus. *Virology* 172, 665-668) and G1L (Sullivan, J. T., Fraser, K., Fleming, S. B., Robinson, A. J. and Mercer, A. A. (1995). Sequence and transcriptional analysis of an orf virus gene encoding ankyrin-like repeat sequences. *Virus Genes*, 9, 277-282). This region (Fig. 8) is another potential site for gene insertion (see Fig. 9).

10 **Example 3 - Identification of Orf Virus Promoters**

Determining the nucleotide sequence of selected regions of the orf virus genome has allowed the identification of a number of orf virus transcriptional promoters, in the first instance by virtue of their similarity to other poxvirus transcriptional promoters, and later 15 by functional assays.

Orf virus early and late promoters are shown in Figure 10. The early promoter E1L (ORF-3) was shown to make mRNA early in the cell cycle (Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia virus-like early transcriptional 20 control sequences flank an early gene in the orf parapoxvirus. *Gene*. 97, 207-212) and the late promoter F1L was deduced to be a late promoter by virtue of its similarity to a vaccinia virus late promoter. The orf virus late promoter is functional in a transient assay. Such assays have been described for instance by (Cochran, M. A., Mackett, M. and Moss, B. (1985). Eukaryotic transient expression system dependent on transcription factors and 25 regulatory DNA sequences of vaccinia virus. *Proc Natl Acad Sci USA*. 82, 19-23). A third promoter F3R, identified as an early-late promoter, is also shown to be functional in a transient assay. The construction of a plasmid pSP-PFlac containing the orf virus late promoter, F1L, and the *E. coli* gene for β -galactosidase (*lacZ*) such that the β -galactosidase gene is under the control of the orf virus late promoter is described in 30 Example 6 and illustrated in Figure 11.

(A) Assessment of Promoter Activity in Transient Assay

To show that the promoter is active in a transient assay, a confluent monolayer of bovine 35 testis cells, in a plastic flask of 25 cm² surface area for the adherence of the cells and suitable for cell culture work, was infected with orf virus at a multiplicity of infection of approximately 10 plaque forming units per cell. Two hours after infection, the plasmid containing the *lacZ* gene linked to the promoter under investigation was introduced into

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orf virus infected bovine testis cells using the liposome mediated transfer technique as described by (Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA*. 84, 7413-7417) and as set forth in Example B. Forty eight hours after infection, 35 μ l of a solution of 5-bromo-4-chloro-3-indolyl- β -D galactosidase (X-gal) at a concentration of 2% w/v in water was added to 1 ml of 1% agarose in cell culture medium which was overlayed onto the cells after the removal of the liquid medium and allowed to form a gel at room temperature (in the range of 15°-25° C). Over the succeeding 24 hours the development of a blue coloration in the cells and in the gel above the affected cells was looked for. The development of a blue coloration greater than that seen in cells treated similarly, but with a plasmid containing the β -galactosidase gene not under control of a transcriptional promoter, indicated that the promoter being tested was active.

In a further aspect of investigating promoter function a quantitative assay for β -galactosidase activity in transiently-infected bovine testis cells is performed. Cells are grown as confluent monolayers in multiwell plastic tissue culture trays containing 24 wells 1.5 cm in diameter. Individual wells are infected with orf virus at a moi of 10 and two hours after infection the plasmid construct containing the promoter linked to the β -galactosidase gene is introduced into the infected cells using the liposome mediated transfection technique described above. Cells are harvested by scraping into a 1 ml volume of phosphate-buffered saline (PBS), collected by centrifugation, washed with PBS and resuspended in a 200 μ l volume of PBS. Cells are disrupted by three cycles of freezing and thawing, centrifuged, and the supernatant retained for the enzyme assay. The assay for β -galactosidase is conveniently performed in 96-well microtitre trays. The reaction mixture of 0.1 ml contains 100mM Na-phosphate, pH 7.3, 1mM MgCl₂, 50mM β -mercaptoethanol, O-nitrophenyl- β -D-galactoside (ONPG) at a final concentration of 1.3mg/ml and a 10-20 μ l aliquot of the cell lysate. The reaction mix is incubated at 37° C for 1 hour and the reaction is terminated by the addition equal volume of 1M NaCO₃. The absorbance of each well is measured at 420 nm using a microtitre plate reader. The absorbance value is proportional to the amount of β -galactosidase activity present in the original extract and this enables the time course of expression and the relative strength of each promoter construct to be determined.

Example 4 - Construction of a Vector Plasmid Suitable for the Insertion of Foreign Genes into the Orf Virus Genome

The choice of non-essential DNA was the region discovered to be deleted in a re-arranged

5 mutant of orf virus and the relevant sequence of nucleotides in this region can be found in Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. *Virology*. 176, 379-389 and in Sullivan, J. T., Fraser, K. M., Fleming, S. B., Robinson, A. J. and Mercer, A. A. (1995). Sequence and transcriptional analysis of an orf virus gene 10 encoding ankyrin-like repeat sequences. *Virus Genes* 9, 277-282 and is shown in Figure 8. The orf virus promoters used were an early promoter, E1L, described in Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. *Virology*. 176, 379-389 and Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia 15 virus-like early transcriptional control sequences flank an early gene in the orf virus. *Gene*. 97, 207-212 and a late promoter F1L (Fleming, S. B., Blok, J., Fraser, K. M., Mercer, A. A. and Robinson, A. A. (1993). Conservation of gene structure and arrangement between vaccinia virus and orf virus. *Virology*. 195, 175-184) as shown in Figure 10. The foreign genes chosen to demonstrate the process of creating a mutated orf 20 virus were the *E. coli* β-galactosidase gene, which has the advantage that when expressed the protein product can be detected by a colour reaction (Miller, J. H. (1972). "Experiments in Molecular Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. Moss, B. (1990). "Poxviridae and their Replication" in *Virology*, Fields et al., eds, 2nd ed. Raven Press, New York, 2079-2111), and the *E. coli* guanyl 25 phosphoribosyl transferase (*x-gpt*) gene which when expressed can be used to select mutants from unmutated virus (Mulligan, R. C. and Berg, P. (1980). Expression of a bacterial gene in mammalian cells. *Science*. 209, 1422-1427). The following is a description of the construction of the vector plasmid. Figures 11 -13 outline the construction in diagrammatic form.

30

(A) Cloning an Orf Virus Late Promoter in Front of the *E. coli LacZ* Gene

In the construction of a mutant orf virus it is an advantage to be able to distinguish mutant virus from unmutated virus by a convenient and rapid assay. Such an assay is provided

35 by inserting the *E. coli* gene for the β-galactosidase enzyme under control of an orf virus transcriptional promoter into the vector plasmid. The late orf virus promoter was identified by determining the nucleotide sequence of a fragment of orf virus DNA designated *Bam*HI F (Fleming, S. B., Blok, J., Fraser, K. M., Mercer, A. A. and Robinson,

A. A. (1993). Conservation of gene structure and arrangement between vaccinia virus and orf virus. *Virology*. 195, 175-184). The sequence of the promoter F1L used in this construction is shown in Fig. 10. A sufficient quantity of the late promoter for the construction can be obtained from the plasmid designated pVU-6 which has been described (Mercer, A. A., Fraser, K., Barns, G. and Robinson, A. J. (1987). The structure and cloning of orf virus DNA. *Virology*. 157, 1-12). A total of 2.62 kb of DNA is deleted from the *Bam*HI F fragment of orf NZ-2 by digesting the plasmid pVU-6, which contains the *Bam*HI F fragment of orf NZ-2 cloned into the plasmid pUC-8 (Viera, J. and Messing, J. (1982). The pUC plasmids, an M13mp7 derived system for insertion 10 mutagenesis and sequencing with synthetic universal primers. *Gene*. 19, 259-268) with *Aval*. This enzyme cleaves the *Sma*I site of the pUC-8 polylinker and six internal *Aval* sites in *Bam*HI E. The *Aval* sites remaining on the vector fragment are end-filled with Klenow DNA polymerase, and religated to give the plasmid pVU-Av6. The plasmid pVU-Av6 is cut with *Bam*HI and *Eco*RI releasing a 725 bp fragment containing the orf 15 virus late promoter. This fragment is cloned into pMLB 1034 (Weinstock, G. M., Berman, M. L. and Silhavy, T. J. (1983). "Chimeric genetics with β -galactosidase in gene amplification and analysis." in Expression of Cloned Genes in Prokaryotic and Eucaryotic Cells, Papas *et al.*, eds. Elsevier, New York, 27-64) which contains a "headless" *lacZ* gene. This cloning places the orf virus late promoter in front of *lacZ* and supplies it with 20 an ATG initiation codon allowing the synthesis of β -galactosidase. The colonies that result from this cloning step give a blue phenotype on X-gal plates facilitating the selection of the required clone. A unique *Bal*I site downstream from the *lacZ* insert of pMLB-1034 is converted to an *Eco*RI site by the following cloning steps. The Tn5 aminoglycoside 3' phosphotransferase gene is released from the plasmid pNEO (Beck, E., 25 Ludwig, A., Aurswald, E. A., Reiss, B. and Schaller, H. (1982). Nucleotide sequence and exact location of the neomycin phosphotransferase from transposon Tn5. *Gene*. 19, 327-336) with *Eco*RI and *Bam*HI. The restriction sites are end-filled with Klenow DNA polymerase and the fragment ligated into plasmid pMLB-PF which had been cut with *Bal*I. Recombinants are selected by plating on kanamycin medium. This creates an 30 *Eco*RI or *Bam*HI site at the position of the original *Bal*I site depending on the orientation of the cloned aminoglycoside 3'-phosphotransferase II (*aph*II) gene. *Bal*I often cuts DNA inefficiently, but the method allows for the selection of the plasmids which have been cut by *Bal*I and have received the insert, consequently becoming modified in the desired manner. The plasmid pMLB-PFneo is cut with *Eco*RI and a 4059 bp *Eco*RI fragment 35 containing the PF-lacZ fusion is cloned into pSP-70 (Melton, D. A., P.A., R., Rebagliati, M. R., Maniatis, T., Zinn, R. and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing

bacteriophage SP6 promoter. *Nucleic Acids Res.* 12, 7035-7056) at the *EcoRI* site to give the plasmid designated pSP-PFlac shown in the diagram Fig. 11.

(B) Cloning of an Orf Virus Early Promoter in Front of the *E. coli X-GPT* Gene

5

In the construction of the mutated orf virus, a means of selecting mutants from non-mutants, from a mixture of both, is required. A method that has been used by others is to utilise the guanyl phosphoribosyl transferase gene of *E. coli*. Resistance is conferred to a metabolic inhibitor, mycophenolic acid, when the gene is expressed in a eukaryotic 10 cell. A method for incorporating this gene into a vector plasmid under the control of an early promoter is described by Falkner, F. G. and Moss, B. (1988). *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J Virol.* 62, 1849-1854 and Boyle, D. B. and Coupar, B. E. (1988). Construction of recombinant fowlpox viruses as vectors for poultry vaccines. *Virus Res.* 10, 343-356.

15 A plasmid designated pVU-5 is used to provide an early orf virus promoter. The plasmid pVU-5 contains the orf virus NZ-2 *BamHI* E fragment cloned into pUC-8 and the construction of this plasmid is described in Mercer, A. A., Fraser, K., Barns, G. and Robinson, A. J. (1987). The structure and cloning of orf virus DNA. *Virology*, 157, 1-12. An early promoter E1L has been described for the putative gene originally designated 20 ORF-3 in pVU-5 by Fraser, K. M., Hill, D. F., Mercer, A. A. and Robinson, A. J. (1990). Sequence analysis of the inverted terminal repetition in the genome of the parapoxvirus, orf virus. *Virology*, 176, 379-389 and by Fleming, S. B., Fraser, K. M., Mercer, A. A. and Robinson, A. J. (1991). Vaccinia virus-like early transcriptional control sequences flank 25 an early gene in the orf parapoxvirus. *Gene*, 97, 207-212; and it is this early promoter that is used in the method described in this application to construct a mutant orf virus. A 503 bp *AluI* A+T-rich fragment shown in the Fig. 12 is cleaved from pVU-5 and cloned into the *HincII* site of the multifunctional plasmid vector pTZ18R described in Mead, D. A., Szczesna-Skorupa, E. and Kemper, B. (1986). Single-stranded DNA "blue" T7 promoter 30 plasmids: a versatile tandem promoter system for cloning and protein engineering. *Protein Eng.* 1, 67-74 giving pSFAlu-6. Plasmid pSFAlu-6 is cut with *DdeI* and the fragments end-filled with Klenow DNA polymerase. The fragments are recut with *HindIII* and a 467 bp *HindIII*-*DdeI* fragment ligated into pSP-70 which is prepared by cutting with *BglII*, end-filling and recutting with *HindIII*. The resulting plasmid pSP-SFP retains the *BglII* site which is reformed during the cloning step. The plasmid pSV-gpt2, containing the *E. 35 coli x-gpt* gene, (Mulligan, R. C. and Berg, P. (1981). Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyl transferase. *Proc Natl Acad Sci USA*, 78, 2072-2076) is cut with *BamHI* and *BglII*. This releases the *x-gpt* gene as a 1788 bp fragment which is then cloned into the *BglII* site of pSP-SFP.

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fusing the orf virus fragment to the *x-gpt* gene giving pSP-SFPgpt32. The plasmid pVU-5 is then cut with *Sma*I and *Sph*I. A 150 bp *Sma*I-*Sph*I fragment containing the early promoter E1L, the sequence of which is shown in Fig. 10, is cloned into pTZ18R between the *Sma*I and *Sph*I sites giving the plasmid pFS-1. The plasmid pFS-1 is cut with 5 *Sph*I and incubated with T4 DNA polymerase. The *aphII* gene is released from the plasmid pNEO with *Eco*RI and *Bam*HI. The *Eco*RI and *Bam*HI sites are end-filled with Klenow DNA polymerase and the fragment ligated into pFS-1. The resulting plasmid 10 pFS-neo3 contains the *aphII* gene flanked by an *Eco*RI site and a *Bam*HI site which lies between it and the early orf virus promoter. A result of these manipulations is that the *Sph*I site distal to the early promoter is converted to a *Bam*HI site. The *aphII* gene and 15 the early promoter lie in a "head-to-head" orientation and may be removed by digestion with *Eco*RI. Next, the plasmid pSP-sSFPgpt32 is cut with *Pvu*II. The *aphII*-early promoter construct was cut out of pFSneo3 with *Eco*RI, end-filled with Klenow DNA polymerase, and ligated into the *Pvu*II site. A plasmid termed FSneo-SFPgpt which 20 contains the early promoter running in the same direction as the 503 bp *Alu*I fragment is selected. The plasmid FSneo-SFPgpt is cut with *Bam*HI and *Bgl*II. This step removes the sequence between nucleotides a and b (Fig. 13) together with the *aphII* gene as a *Bam*HI-*Bgl*II fragment. The vector fragment is subjected to electrophoresis in an agarose gel and then purified using the powdered glass milk method described by (Vogelstein, B. and 25 Gillespie, D. (1979). Preparation and analytical purification of DNA from agarose. *Proc Natl Acad Sci USA.* 76, 615-619) and the free *Bam*I and *Bgl*II termini ligated together fusing the early promoter to the *x-gpt* gene. The net result of the manipulations described in steps 4, 5, 6, and 7 (Fig. 13) was to replace the sequence between nucleotides a and b in pSP-SFPgpt32 with the FS promoter forming pFS-gpt.

25

Example 5 - Identification of a Non-essential Region of the Orf Virus Genome and Insertion of this Site into a Plasmid

A gene coding, potentially, for a peptide of 159 amino acids was found from the 30 sequencing of the 4.47 kb *Bam*HI E fragment which spans the ITR junction of the orf virus genome. This was termed E3L (ORF-PP) and shows homology to an open reading frame in retroviruses (Mercer, A. A., Fraser, K. M., Stockwell, P. A. and Robinson, A. J. (1989). A homologue of retroviral pseudoproteases in the parapoxvirus, orf virus. *Virology.* 172, 665-668) and to *E. coli* dUTPase (McGeoch, D. J. (1990). Protein 35 sequence comparisons show that the 'pseudoproteases' encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase family. *Nucleic Acids Res.* 18, 4105-4110). A spontaneous mutant of orf virus isolated in the laboratory was found not to contain the E3L gene due to a complex rearrangement involving the deletion of part of

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the *Bam*HI E fragment and duplication of DNA segments from the opposite end of the genome at that locus. The E3L gene is therefore non-essential and was chosen as a target for the insertion of foreign DNA and to demonstrate that orf virus could tolerate the insertion of a foreign gene. A 2587 bp *Sma*I-*Bam*HI fragment (Fig. 14) containing the 5 unique region of NZ-2 *Bam*HI E is cut out of pVU-5 and cloned into pSP-70 cut with *Pvu*II and *Bgl*II. The resulting plasmid, pVU-DL100 contains a unique *Nco*I site that lies between the coding sequence of the E3L gene and its promoter.

Example 6 - Insertion of the *E. coli X-GPT* and *Lac Z* Gene Constructs into pVU-DL100
10 to Create a Vector Plasmid

Plasmid pVU-DL100 is cut with *Nco*I and end-filled with Klenow polymerase. The E3L-gpt construct is cut from pFSP-gpt with *Eco*RI and *Dra*I, end-filled with Klenow polymerase and ligated into pVU-DL100 at the *Nco*I site. Ligation of the end-filled 15 *Eco*RI site of the insert to the end-filled *Nco*I site on the plasmid creates an *Eco*RI site upstream of the early promoter. The insert is recovered in two orientations, pVU-DL101 with the *x-gpt* gene running in the opposite direction to the pseudoprotease gene and pVU-DL102 with the *x-gpt* gene running in the same direction as the pseudoprotease gene. The F1L-lac construct is cut out of pSP-PFlac with *Eco*RI and cloned into the *Eco*RI sites of 20 both pVU-DL101 and pVU-DL102. Four plasmids with different orientations of the inserted fragments are recovered from the cloning but only two, pVU-DL104 derived from pVU-DL101, and pVU-DL106 derived from pVU-DL102 which contain the E3L-gpt and F1L-lac in the "back-to-back" orientation are used for transfection experiments.

25 Example 7- Constructing a Chimeric Gene Expressing the *T. ovis* 45W antigen.

A 64 bp fragment of the VEGF like-gene from orf virus NZ-7 (Lyttle, D. J., Fraser, K. M., Fleming, S. B., Mercer, A. A. and Robinson, A. J. (1993) Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. *J Virol.* 68, 84-92) 30 containing five 3' prime terminal codons, the translational termination codon TAA, and a poxvirus transcriptional terminator sequence 5TNT, was amplified using a pair of oligonucleotide primers designed to provide a *Bgl*II and a *Nco*I restriction site flanking the amplified sequence. This fragment was digested with *Bgl*II and *Nco*I and ligated into the vector pSL301 (Brosius, J. (1989) Superlinkers in cloning and expression vectors. *DNA* 8, 759-777) cut with *Bgl*II and *Nco*I to form the plasmid ptov1. A DNA fragment 35 containing the *aph*II gene and the F1L and F3R promoters of orf virus was amplified by PCR using specific primers which introduced a *Mlu*I site at one end and a *Nsi*I and *Eco*RI site at the other end. One portion of the amplified product was digested with *Mlu*I and

*Eco*RI and ligated into ptov1 cut with *Mlu*I and *Eco*RI to create the plasmid ptov2. A second portion was digested with *Mlu*I and *Nsi*I and ligated into ptov1 to form the plasmid ptov3. The steps showing this construction are illustrated in Figure 15.

5 The *aphII* gene was removed from the plasmid ptov2 by digesting with the restriction enzymes *Bam*HI and *Bgl*II, purifying the vector fragment and re-ligating the free ends to form the plasmid ptov5. The DNA sequence encoding the *Taenia ovis* 45W antigen fragment was removed from the plasmid pGEX 45W (Johnson, K. S., Harrison, G. B. L., Lightowers, M. W., O'Hoy, K. L., Cougle, W. G., Dempster, R. P., Lawrence, S. B.,
10 Vinton, J. G., Heath, D. D., and Rickard, M. D. (1989). Vaccination against ovine cysticercosis using a defined recombinant antigen. (*Nature* 338, 585-587) by digesting with the restriction enzymes *Eco*RI and *Bam* HI and ligating it into ptov5 cut with *Bam*HI and *Eco*RI to form ptov6. This placed the DNA sequence encoding the 45W antigen fragment under the control of the orf virus PF3R promoter and supplied it with
15 translational and transcriptional termination sequences. These steps are illustrated in Figure 16.

A 73 bp fragment from the 5' portion of the VEGF-like gene from orf virus NZ-7 encoding the presumptive secretory leader sequence was amplified with specific primers
20 which introduced a new initiation codon, a *Pst*I and an *Eco*RI restriction site into the amplified DNA fragment. The amplified fragment was digested with *Pst*I and *Eco*RI and cloned into ptov3 cut with *Nsi*I and *Eco*RI to create the plasmid ptov4. The plasmid ptov4 was digested with *Bam*HI to remove the *aphII* gene, purified by agarose gel electrophoresis and religated to form the ptov7. The DNA sequence encoding the 45W
25 antigen fragment was removed from the plasmid pGEX 45W by digesting with the restriction enzymes *Eco*RI and *Bam* HI and ligating it into ptov7 cut with *Bam*HI and *Eco*RI to form ptov8. This placed the 45W antigen fragment under the control of the orf virus PF3R promoter and supplied a 5' protein secretory leader sequence in addition to the 3' translational and transcriptional terminators present in ptov6. These steps are illustrated
30 in Figure 17.

The plasmid pVU-DL101 was cut with *Eco*RI and an oligonucleotide linker containing a *Bam*HI and a *Nco*I restriction site was ligated in to form the plasmid pVU DL101L4. This plasmid was then digested with *Bam*HI and *Nco*I to allow the insertion of both
35 versions of the chimeric 45W gene from ptov6 and from ptov8. The resulting plasmids were designated pVU-dl45W (from ptov6) and pVU-dl45W1 (from ptov8). These steps are illustrated in Figure 18.

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A promoterless *lacZ* gene was cleaved out of the plasmid pVUsp-PF2lac, a derivative of pSP PFlac illustrated in Fig. 11 by digestion with BamHI and BglII. In this latter version of the plasmid, the F1L promoter fragment has been truncated to 100 base pairs and a BglII restriction site introduced distal to the *lacZ* gene. The *lacZ* fragment was gel purified
5 and ligated into both pVU-DL45W and pVU-Dl45Wl at a unique BamHI site. This placed the *lacZ* gene under the control of the F1L promoter and completed the construction of the transfer vectors for introducing the *T. ovis* 45W gene into the orf virus genome. These steps are illustrated in Figure 19.

10 The same oligonucleotide linker containing the BamHI and a NcoI restriction sites was ligated into the plasmid pVU-DL102. This plasmid contains the x-gpt gene cloned in the opposite orientation to that in pVU-DL101 (Fig 14). Cloning steps parallel to those described for pVU-DL101 were subsequently performed and the transfer vectors which were generated were designated pVU-DL45W6lac and pVU-DL45W8lac. These
15 contained the same sequences as pVU-DL45Wl lac and pVU-DL45Wllac respectively, but differed in that the entire inserted region was in the opposite orientation to that illustrated for these plasmids in Fig. 19.

Example 8 - Transfection Protocol

20 Primary bovine testis (BT) cells were grown in monolayer cultures in Eagle's Minimal Essential Medium (MEM; Sigma Cat. No. M0643) supplemented with lactalbumin hydrolysate (5 g/L) and 5% foetal calf serum. Medium for selecting orf virus transformants expressing x-gpt contain mycophenolic acid, 25 µg/ml, xanthine, 250 µg/ml, hypoxanthine, 15 µg/ml, aminopterin, 1 µg/ml, thymidine, 5 µg/ml and 2% foetal calf serum. Lactalbumin hydrolysate was omitted from the selective medium and replaced with additional non-essential amino acids (MEM non-essential amino acid mixture, Sigma Cat. No. M2025).

30 BT cells were grown as monolayers in a suitable cell culture vessel. Twenty-four hours prior to infection, the cell growth medium was replaced with the selective medium containing mycophenolic acid. The cells were infected with orf virus, strain NZ-2, (moi 0.05 - 0.1) and the virus allowed to adsorb for 1 hour. Cell monolayers were washed 2 times with opti-MEM serum-free medium, (Life Technologies Inc, Gaithersburg, MD
35 U.S.A.) to remove residual foetal calf serum, and drained. A 1.0 ml volume of opti-MEM containing 10. µl Lipofectin reagent (Life Technologies Inc, Gaithersburg, MD, U.S.A.) and approximately 2.0 µg plasmid DNA diluted according to the suppliers instructions was added to each flask and incubated overnight. Following this overnight adsorption

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step, 5.0 ml of selective medium containing 2% foetal calf serum was added and the incubation continued until cytopathic effect (CPE) was observed approximately 3 - 5 days post-infection.

5 Cell monolayers were scraped from the flask, deposited in the bottom of a centrifuge tube by low speed centrifugation, washed with phosphate buffered saline (PBS) and resuspended in PBS. A suitable tissue culture vessel was seeded with BT cells to produce a confluent monolayer. Routinely, 60mm diameter polystyrene dishes were used, seeded with 1.5×10^6 cells per dish and incubated in a CO₂ atmosphere to maintain a pH of

10 around 7.2. The culture medium was removed and 0.5 ml of an appropriate dilution of orf virus in PBS was added and incubated for one hour at 37°C. Dishes were tipped at 15 min intervals to ensure an even distribution of fluid. At the end of this time the inoculum was removed and growth medium containing 1% agarose added. After five days, the time when plaques usually become visible, X-gal was added to the dish in a 1% agarose

15 overlay and incubated a further 12 hours for colour development to occur. Single plaques are picked, resuspended in PBS and inoculated into a partially drained cell culture vessel which had been seeded with 2×10^5 cells and grown to confluence as described. One ml of medium was added to each well and incubation at 37°C continued until a complete cytopathic effect was observed. The cell culture vessels were placed at -20°C until the

20 contents were frozen after which time they were thawed. The cell lysates were used as a source of virus, for further plaque purification, and of viral DNA for hybridisation. Viral DNA was prepared from cytoplasmic extracts of BT cells by the method of Moyer, R. W. and Graves, R. L. (1981). The mechanism of cytoplasmic orthopoxvirus DNA replication. *Cell.* 27, 391-401. The isolated DNA was digested with restriction enzymes

25 to confirm the insertion of the foreign genes. Frequently, the first plaque purification step fails to remove all the wild type virus and a series of plaque purification steps may be performed in order to obtain a pure culture of mutated virus. Bulk cultures of virus are grown in 150 cm² tissue culture flasks and the virus purified by the method described in Robinson, A. J., Ellis, G. and Balassu, T. (1982). The genome of orf virus: restriction

30 endonuclease analysis of viral DNA isolated from lesions of orf in sheep. *Arch Virol.* 71, 43-55. DNA is extracted from the purified virions by the method described in Balassu, T. C. and Robinson, A. J. (1987). Orf virus replication in bovine testis cells: kinetics of viral DNA, polypeptide, and infectious virus production and analysis of virion polypeptides. *Arch Virol.* 97, 267-281.

Example 9 - Assessment of Orf Virus Modification

In order to determine whether or not the viruses recovered from the transfections and plaque purifications were modified to carry the inserted genes, DNA was prepared from 5 infected cells and tested by hybridisation by methods well known to those skilled in the art, for example, Merchlinsky, M. and Moss, B. (1989). Resolution of vaccinia virus DNA concatemer junctions requires late-gene expression. *J Virol.* 63, 1595-1603. In the preparation of mutated orf virus DNA for these tests, a 100 µl aliquot of orf virus-infected BT cells in PBS was centrifuged for 30 min at approximately 12,000g. The cell pellet was 10 resuspended in 50 µl 0.15M NaCl, 20mM Tris, 10 mM EDTA, pH 8.0. A 250 µl volume of 20mM Tris, 10mM EDTA, 0.75% SDS containing a protease at an appropriate concentration (e.g. Proteinase K at 0.5 mg/ml) was added to each sample and incubated at 37° C for 3 hours. The samples were extracted with an equal volume of phenol:chloroform (1:1) before precipitation with ethanol. Following centrifugation the 15 ethanol-precipitated DNA was redissolved in 50 µl TE. The material harvested from the various passages was subjected to the hybridization procedure with a specific *x-gpt* probe. A positive result can be obtained with pVU-DL106 for the transfection two hours post-infection as early as passage one. An alternative procedure that was used to detect heterologous DNA markers in recombinant virus was to amplify DNA sequences by the 20 polymerase chain reaction using primers specifically designed to amplify the foreign DNA sequences. Other transfections may require further passages for the detection of recombinant viruses. Transfections performed with the plasmid pVU-DL106 at two hours allowed CPE to be detected at three days post-inoculation at passage three and the detection of mutated virus containing the *x-gpt* gene as determined by DNA-DNA 25 hybridization. A qualitative assay for β-galactosidase activity using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was used to detect mutated orf virus containing the β-galactosidase gene.

Example 10 - Construction of a vector plasmid suitable for the insertion of foreign genes 30 into the region of the orf virus genome corresponding to the orthopoxvirus ATI-region

The intergenic region between the RNA polymerase subunit gene, rpo 132 and the open reading frame of the presumptive gene (H)I1L was identified as a suitable target site for the insertion of foreign DNA. The region is 90 nucleotides in length and lies between 35 two converging transcriptional elements one of which, rpo 132, is an essential gene. A plasmid, PB-23ΔSal, which contains a sequence of 1.6 kilobases extending into the unsequenced region upstream of position 1 shown in the sequence illustrated in Figure 5 and terminating at the *Pst*I site at position 178 was used as the template in a PCR cloning

- 26 -

reaction. A sequence of 1.0 kb was amplified from it using the primers zxs-1 GATCCCGCTCGAGAACTTCAA (forward) which is complementary to a sequence identified in PB-23 Δ Sal that contains an existing *Xba*I restriction site and zxs-2 GTCAGATCTATGCATAAAAATTCGCATCAGTCGAGATA (reverse) which

5 introduces a *Bgl*II, a *Nsi*I and an *Apo*I restriction site. The amplified fragment was purified by electrophoresis on a 1% agarose gel and digested with the restriction enzymes *Xba*I and *Bgl*II. The purified fragment was ligated then into the plasmid pSP-70 at the corresponding *Xba*I and *Bgl*II sites creating the plasmid pTvec1. This cloning step also introduced a poxvirus transcriptional termination signal (STNT) into the vector.

10

A second fragment comprising the sequence located between nucleotide positions 66 and 1069 (Fig 5) was amplified with the primers zxs-3 GACATGCATCAGTGCCATGGAATTCTCGCGACTTTCTAGC (forward) which introduces *Nsi*I, *Nco*I and *Eco*RI restriction sites and zxs-4

15

GACGGATCCGTATAATGGAAAGATT (reverse) which introduces a *Bam*HI restriction site. The amplified fragment was digested with the restriction endonucleases *Bam*HI and *Nsi*I and purified in the same manner as the first fragment. The purified fragment was then cloned into pTvec1 which had been cut with *Nsi*I and *Bgl*II. The resulting plasmid pTvec50 contains a series of restriction sites and a transcriptional 20 termination signal which are available for further cloning steps. These restriction sites are *Apo*I, *Nsi*I, *Nco*I and *Eco*RI. The sequence of the primers, the restriction sites and the sequence of the modified intergenic region are shown in Figures 20A and 20B. The cloning steps involve in the construction of ptvec50 are illustrated in Figure 21.

25

A *lacZ* gene under the control the orf virus late promoter PF1L was cleaved out of the plasmid pVUsp-PF2lac with *Eco*RI. The fragment was gel purified and ligated into the *Eco*RI site of pTvec50. Recombinant plasmids containing the *lacZ* gene in both possible orientations were recovered and designated pTvec50lac-1 and pTvec50lac-2. The cloning steps involved in the construction of pTvec50lac-1 and pTvec50lac-2 are illustrated in 30 Figure 22. This completed the construction of a transfer vector designed to introduce the foreign gene *lacZ* into the intergenic site between the open reading frames of rpo 132 and (H)I1L shown in Fig 5 .

In this example the *xgpt* gene was not included in the transfer vector and consequently 35 selection of recombinant orf virus expressing *xgpt* by growth in the presence of mycophenolic acid was not able to be used as a selection method. Virus recombinants were selected by using *lacZ* expression as the primary method for identifying

recombinants containing an insertion in the ATI region. The following variation of the method described in Example 8 was used.

Primary bovine testis (BT) cells were grown in monolayer cultures in Eagle's Minimal
5 Essential Medium (MEM); (Sigma Cat. No. M0643) supplemented with lactalbumin hydrolysate (5 g/L) and 5% foetal calf serum. Prior to infection the cell growth medium was removed and the cells washed briefly with phosphate buffered saline (PBS) to remove residual serum. The cells were infected with orf virus, strain NZ-2, (moi 0.05 - 0.1) and the virus allowed to adsorb for 1 hour. Cell monolayers were washed 2 times with optimised
10 MEM serum-free medium, (Life Technologies Inc, Gaithersburg, MD, U.S.A.) to remove non-adsorbed virus and residual foetal calf serum, and drained. A 1.0 ml volume of optimised MEM containing 10 µl Lipofectin reagent (Life Technologies Inc, Gaithersburg, MD, U.S.A.) and approximately 2.0 µg plasmid DNA diluted according to the suppliers instructions was added to each flask and incubated overnight. Following this overnight
15 adsorption step, 5.0 ml of selective medium containing 2% foetal calf serum was added and the incubation continued until cytopathic effect (CPE) was observed approximately 3 - 5 days post-infection.

Cell monolayers were scraped from the flask, deposited in the bottom of a centrifuge tube
20 by low speed centrifugation, washed with PBS and resuspended in PBS. The resuspended cells were subjected to three cycles of freezing and thawing and sonicated briefly. The virus titre of the harvested culture was determined and the material plated on fresh dishes of BT cells at a dilution calculated to give approximately 2000 virus plaques per dish. Sufficient material was plated to screen 50,000 plaques (25 dishes). The infected
25 monolayers were grown under an a 1% agarose overlay and after 5 days incubation when plaques became visible, X-gal in a 1% agarose overlay was added to the dishes and incubated a further 12 hours for colour development to occur. At this stage, any coloured plaques which had appeared were picked and treated as described in Example 8. Further purification of the recombinant virus was achieved by repeated cycles of plating and picking single, coloured plaques until a pure culture of *lacZ* positive virus was obtained.
30

APPLICATION OF THE INVENTION

In accordance with the present invention there is provided a parapoxvirus vector,
35 specifically an orf virus vector, containing exogenous DNA. The exogenous DNA may encode an antigen capable of inducing an immune response or may encode a heterologous polypeptide of which expression is desired.

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The vectors of the present invention therefore have particular applications in the expression of heterologous polypeptides and antigens. The capacity to express antigens make these vectors particularly suitable for use in vaccines.

- 5 Orf virus vectors have a number of advantages over vaccinia virus vectors. Orf virus has a relatively narrow host range compared to vaccinia. This reduces the vaccinia associated risks of cross-species infection and spread of disease. A further advantage is that orf virus is less virulent than vaccinia in man, reducing the risks of febrile response and lesions.
- 10 It will be appreciated that the above description is provided by way of example only and that the invention is limited only by the scope of the appended claims.

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CLAIMS:

1. A parapoxvirus vector comprising a parapox virus containing exogenous DNA.
- 5 2. A vector as claimed in claim 1 wherein the parapox virus is orf virus.
3. A vector as claimed in claim 1 or claim 2 wherein the exogenous DNA encodes at least one gene product.
- 10 4. A vector as claimed in claim 3 wherein one gene product encoded is an antigen capable of inducing an immune response.
5. A vector as claimed in claim 4 wherein the antigen is selected from the group consisting of HIV envelope protein, herpes simplex virus glycoprotein, *Taenia ovis*,
15 *Echinococcus granulosis* antigens, *Trichostrongylus* antigens, *Haemonchus* antigens, *Ostertagia* antigens and combinations thereof.
6. A vector as claimed in claim 5 wherein the antigen is a *Taenia ovis* antigen selected from the group comprising *Taenia ovis* 45W, 16kd, 18kd antigens and combinations
20 thereof.
7. A vector as claimed in any one of claims 3 to 6 wherein the exogenous DNA further encodes at least one product which is a biological effector molecule.
- 25 8. A vector as claimed in claim 7 wherein the biological effector molecule is selected from the group comprising γ interferon, IL-1, IL-2, IL-1 β , IL-4, IL-5, IL-6, IL-12 and combinations thereof.
9. A vector as claimed in claim 8 wherein the biological effector molecule is selected
30 from the group comprising IL-1, IL-2, IL-12 and combinations thereof.
10. A vector as claimed in any one of claims 3 to 9 wherein the exogenous DNA further encodes at least one peptide moiety expressed as a hybrid or chimeric protein with a native virus protein.
35
11. A vector as claimed in any one of claims 1 to 10 wherein the exogenous DNA is incorporated in one or more non-essential regions of the virus genome.

- 34 -

12. A vector as claimed in claim 11 wherein the non-essential regions are selected from the non-essential regions identified in accompanying Figures 2, 3, 5 and 7.

5 13. A vector as claimed in claim 11 or claim 12 wherein the non-essential region is from nucleic acids 11 to 16 in the sequence of Figure 5 or from nucleic acids 2226 to 2286 in the sequence of Figure 9.

14. A vector as claimed in any one of claims 1 to 13 wherein the exogenous DNA is under the control of a poxvirus promoter.

10

15. A vector as claimed in claim 14 wherein the poxvirus promoter is an orf virus promoter.

15

16. A vector as claimed in claim 15 wherein the orf virus promoter is selected from the group consisting of E1L, F1L and F3L as set forth in Figure 10.

17. A vector as claimed in any one of claims 3 to 16 wherein the exogenous DNA further encodes a reporter gene.

20

18. A vector as claimed in any one of claims 3 to 17 wherein the exogenous DNA further encodes a selectable marker.

19. A fragment or variant of a vector as claimed in any one of claims 4 to 18 having equivalent immunological activity thereto.

25

20. A vaccine comprising a viral vector according to any one of claims 1 to 18 or a fragment or variant thereof as claimed in claim 19.

30

21. A vaccine as claimed in claim 20 which further comprises a pharmaceutically acceptable carrier and/or adjuvant therefor.

22. A host cell incorporating a vector as claimed in any one of claims 1 to 18.

23. A host cell according to claim 22 which is a eukaryotic cell.

35

24. A host cell according to claim 22 or claim 23 which is a bovine testis cell or an ovine testis cell.

- 35 -

25. A method for producing recombinant parapoxvirus vectors comprising transfecting a vector of any one of claims 1 to 18 into a selected host cell infected with orf virus; selecting a recombinant virus; and optionally purifying the selected virus.

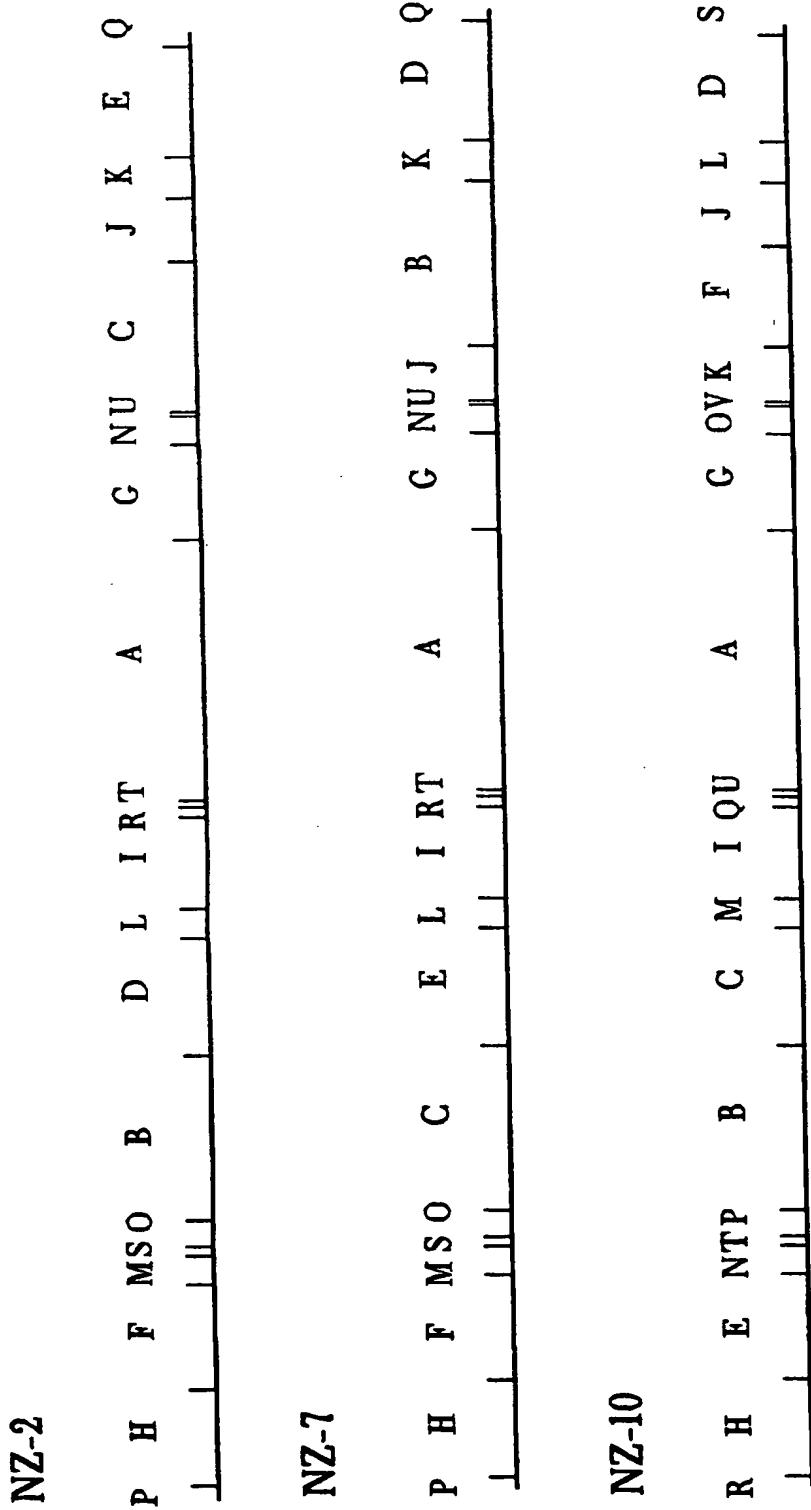


Figure 1. KpnI Maps of Orf Virus Genomes.

Figure 2. Nucleotide sequence of part of the KpnI E fragment of orf virus strain NZ-2.

1	11	21	31	41	51	61	71	81	91
	GCCCTCAC	GCGCTCTCT	CGGAGCTCAC	GGTCTCTGCC	TCCCCTGGGA	CCTCGACGG	CTTCCGGCTC	CACGTCTCT	CAGGACAC
101	111	121	131	141	151	161	171	181	191
	CTCGCCGCC	TCGCGCCG	CGACCTCTTC	TCCGGCTGGA	TAATGGCC	CGCGACGCC	CCCGAACCCG	CGGACTCTC	CTGAGGCCAC
201	211	221	231	241	251	261	271	281	291
	CCGGGCTGC	TGTAACGCT	CTTCCTCGG	CGCTTCCTGC	GccccGCTCC	CGGCGGCTC	CGGCCGCTG	CGCCGCTGC	CTGGCTGCC
301	311	321	331	341	351	361	371	381	391
	TGCGGGCCG	CTCGGAAAC	TGCGGACTAG	TGGGCTGAA	CTGGCTGCC	GCGGACGGG	CCGGCGCT	TCCGGGGC	TGCGGAGAC
401	411	421	431	441	451	461	471	481	491
	GCTGGCGAG	CTCCCCGGG	CGGAAAGCT	CGCGCTGCC	CTCGAGCTCG	GGTTGGGCC	CGAGGACCCG	GAGCTAGGC	CGGACCCCC
501	511	521	531	541	551	561	571	581	591
	GAGAGCCAC	TGGCACAGA	CATCCACATC	CAGACCTCG	ACCTGGGGCA	CTCGGGAGAC	CCCAAAGGGC	GGCGACTCG	CTGAAACAGG
601	611	621	631	641	651	661	671	681	691
	GCCACGGGC	CGCGAACCTC	CGCGCGGC	GGTGGCGAC	CGCGTGACG	CGCGGCTGC	CCGGGAGCCG	GCACGGCTC	GGCGGCTCG
701	711	721	731	741	751	761	771	781	791
	GTGGACCTG	CTGGCTGGG	TGGCGGGT	GACGGCTC	GGCGTGCTG	CGGTTTCACT	GCTCGGGCC	GGCGTGGGG	TACGTTACCG
801	811	821	831	841	851	861	VEGF-2	→	891
	CGGGCGGC	TGCGCGCTA	GCCCCGAA	ATGTAATTAA	TAACGCCAA	CTTTTAAGG	TGAGGGCCA	TGAAGTGT	CTAGTAGCCG
901	911	921	931	941	951	961			991
	TGTGCTGCA	CCAGTATCTG	CTGAGCGGG	ACAGAACAC	GAAGGATGG	TCCGAAGTGC	TGAAGGGCAG	CAGTGCAG	TCTCTGTC
1001	1011	1021	1031	1041	1051	1061	1071	1081	1091
	TGTAACGGAG	ACGCCACCG	AEGTCACTTC	TCAAGGGTTC	AACCCCGT	GTGTCAGCT	GATCCGATGC	GGGGGTGCT	GCAACGACGA
1101	1111	1121	1131	1141	1151	1161	1171	1181	1191
	TCGTCACCCA	CGGAAGAGT	AAACGTGACG	ATGGAACCTC	TGGGGCGTC	AGGCTCCCGT	AGTAACGGGA	TGCAACGCT	GGGTTGTA

Fig 2.1

1201 AATGGGATTG TAGACCAAGCA TTAGCACACCA CGCCACCGAC GACCACAGG CCGGCCAGAA GACGCCCTTA GAACCTTTA TGGACCGCAG ATCCAAACCA
 1211 1221 1231 1241 1251 1261 1271 1281 1291
 1301 TGATGGCATC AGGTCTATGG GAAGAGGGC CACGGAGCA AAGTAAAAA GGACCGCTTA GCAGTGAGA CCCTCCGGCC GCAGCCGGG ACACCCACCA
 1311 1321 ITR junction 1351 1361 1371 1381 1391
 1401 CCCGGCTTC ACCGGCAGA CGCCAAACCC GCAGCCAAACA AGCATCG
 1411 1421 1431 1441

Fig 2.2

Figure 3. Nucleotide sequence of part of the KpnI D fragment of orf virus strain NZ-7.

1 11 21 31 41 51 61 71 81 91
 | | | | | | | | |
 GGGTCCAC CGCTCTCT CGAGCTCAC GGTCTGTC TCGGCCGGA CCTGGACCC CTTCCGCTG CGGGCACCC CATGGACAC
 101 111 121 131 141 151 161 171 181 191
 | | | | | | | | |
 CTGGCGGC TGGCGCGC CGACGTCGTC AGCGCTGGA TAACTCGGC TCAAGAGCC CGGACCGCC CACTCTCG AGGCCACCC CGCGCGCC
 201 211 221 231 241 251 261 271 281 291
 | | | | | | | | |
 GGCTGGCT ACGACGCTCT CCTCGGCCG TTCTGGCC GGTGGCCG GCGGCCGC GCGGGGGG CGGGCTGG CGTGCGCTG GGTGGGCC
 301 311 321 331 341 351 361 371 381 391
 | | | | | | | | |
 CGGGTGGCT CGGGAACTGC GACCTGGGG TGCTGAACCG CTGGCACGGG GACGCCGCC GACGGCTCG CGCTGGCTC CGGGGGCTG GCGGAAAGCC
 401 411 421 431 441 451 461 471 481 491
 | | | | | | | | |
 TGGGGAGCT GCCGGCGCG GACAAGCTG CGCTCGGG CGAGCTAGGG GTGGACCGG AGCACCCGG AGTGAACCGG GACCCCCG GCGGGCGA
 501 511 521 531 541 551 561 571 581 591
 | | | | | | | | |
 GGGGGCTC GCACAGAAC TCGACATCA GACGCTGAC CTAGGGACT GCGCGACCC CAAGGCCCG CGACTGGCG TGGCGCTGT GAACAGGCC
 601 611 621 631 641 651 661 671 681 691
 | | | | | | | | |
 CGAACTGGCC GCTGGCGGC GTCGGACCC CGGGGGCGG CGTGTACGG CGGGGTGCC ATTRGCTGC TGGCGCGGC AGGGCCCGC AGGGGGCGC AGCCGGCGT
 701 711 721 731 741 751 761 771 781 791
 | | | | | | | | |
 GAGCGCTCT GCTGGCGGC CGGGGGCGG CGGGGGCGG CGGGGGCGG ATTRGCTGC TGGCGCGGC GCTAAGATA CGGGTTAGAT ACTCAAAGTC
 801 811 821 831 841 851 861 871 881 891
 | | | | | | | | |
 TATCCAGACA CTTAGAGCT AACTTGAGT AAAATTGTA AATACTTAAGC CCAAAATTTC GATAGTTGTT AGGAATAATA TAACATTTT AAAACGTAT
 901 911 921 931 941 951 961 971 981 991
 | | | | | | | | |
 VBGF-7 → | | | | | | | |
 CACCGCATTA AAGTAACAG CTACCTTACA AGTGTGTTT GCATTGTTAA TATGTATGTA TAATTGCCA GAAATGGGTGCTCAGAGTA TGATTCACT
 1001 1011 1021 1031 1041 1051 1061 1071 1081 1091
 | | | | | | | | |
 CCTTCACCA ATAGCTGGAT GGCGACATA GACAAAGTA GTTGTAAACC TAGAGATACT GTTGTAAATT TGGAGAAGA ATATCCAGAA AGCACTAAC
 1101 1111 1121 1131 1141 1151 1161 1171 1181 1191
 | | | | | | | | |
 TACAAATAAA TCCCAGGCGC GTAACTGTA AACGATCAG TGGTTGCTG AACGGTACG TACAGCGGT GAMACAGAA ATACAAGCT
 | | | | | | | | |

Fig 3.1

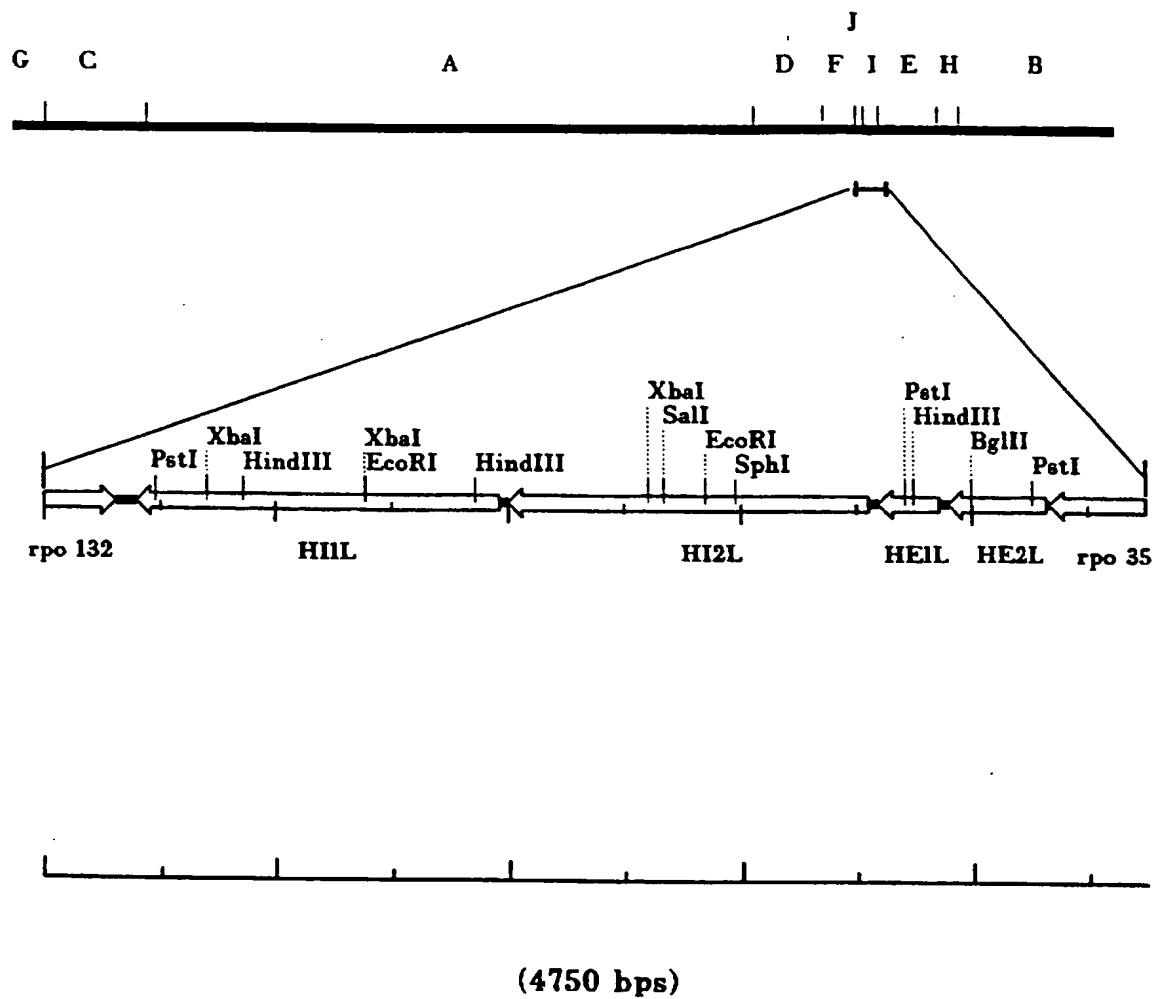
5/34

1201 1211 1221 1231 1241 1251 1261 1271 1281 1291
 AACAGTTCA GTAAACGGCG TGTCTAGTC GTCGTGACT AATAGTGTG TATCTACTAA CCTTCAGA ATAGTGTG CAGAACAC AAAGTGCAT
 1301 1311 1321 1331 1341 1351 1361 1371 1381 1391
 TGTATGGTA GAAACGAC ACCACTAGGG AACCTGACG ATAACTAATA ACAAAATG TTATTTTG TAATACTTA ATTATCAC
 1401 1411 1421 1431 1441 1451 1461 1471 1481 1491
 ACTTTACAAT ATATCAAA ATAATTCGG TCCCCGGAG GCTGCAGGT GTGACGCCGC TGTGTACAC ACTGGTATT CGATTCAAGT TCACAAACG
 1501 1511 1521 1531 1541 1551 1561 1571 1581 1591
 CACTAACTA GTTGTGGT ACCCTGTTT CAACATCAA TCTTACCTT CCGTGACAG AGCTAAACT TGAAACCAT ATTTTAAAG
 1601 1611 1621 1631 1641 1651 1661 1671 1681 1691
 TATATTAAC AATTAACCT ACACTCAC ACCACAAAC ACCACACCA CAACCAAAC CGCATGAGA TTAATTTCT TACTTACCG TAACACTCA
 1701 1711 1721 1731 1741 1751 1761 1771 1781 1791
 TGTGTACAT CAACCCATCA GAGGACTCTG AGTCGACTA ATGGGGCAA ACGGGAACCC AGGGGGACA TAATCTGGA GATCTCGG AGAACCCGT
 1801 1811 1821 1831 1841 1851 1861 1871 1881 1891
 CAAGGACATC TCTAGGCTA ACGGGTGTT GTCAATCCC CTGTGTCA TCTCACCA CATTGTGACC GTGCAAGG ACACATCAA ACTGCCGAT
 1901 1911 1921 1931 1941 1951 1961 1971 1981 1991
 GTGGAAAGAT TCACTGAAAC ACCCTGGTC TAAACAT GTCCGATCT AGACAGAGT TTATGATTC TACGACAC CACGTGCAA TAGGACCTC
 2001 2011 2021 2031 2041 2051 2061 2071 2081 2091
 GTAAATTGTT AGACTGAAAC TCTCTGGTC TAAACAT GTCCGATCT AGACAGAGT TTATGATTC TACGACAC CACGTGCAA TAGGACCTC
 2101 2111 2121 2131 2141 2151 2161 2171 2181 2191
 TCTTCTTGT CACTCCCTTG TCTTAGATGA GTTATACAG ACATGATGA TGTGTGCCC GGCGGGGC GGCGGGCTG GGGGGGGC TGTCTGGCGC
 2201 2211 2221 2231 2241 2251 2261 2271 2281 2291
 GGCGGGCC CGCTGGCCG GACTGGCCG CGCGT-GCGS CGGGGGGC GCGGGGGT AGGGGGCC CGGGGGGC GCGGGGGC GCGGGGGC
 2301 2311 2321 2331 2341 2351 2361 2371 2381 2391
 CGGACAGGC GCACGGAGC AAGTGAAGA AGGACGGCT AGGAGTGTG ACCCTGGCC CGGGGGCC AGCCCCACA CCCGGCTTCC ACCGGCCAGA
 2401 2411 2421 2431 2441 2451 2461 2471 2481
 CGCACACCC ACAGCCACCA AGCATGCAAC CCGGGCGG CAGGTGCTC GGGGGCTCG CGGGGGCC CGGGGGCC TGGGGCTTCC TGGGGGGG

Fig 3.2

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FIGURE 4. HindIII Map of Orf virus NZ-2 showing the location and orientation of the reading frames for the putative genes, rpo132, (H)I1L, (H)I2L, (H)E1L, (H)E2L, and (H)E3L (rpo35).



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Figure 5. Nucleotide sequence of genes H11L, H12L, HELL and HE2L from orf virus strain NZ-2 showing potential insertion sites.

```

1      11     21     31     41     51     61     71     81     91
|      |       |       |       |       |       |       |       |
GACTGATGG AACGCCGG CGACCCGGG ACTAACCTA TCTCGACTGA TCGGAACCG CGACCTCTCG CGACTTCTCA GCTTCCTAGA CTTATGCTAC
101    111    121    131    141    151    161    171    181    191
|      |       |       |       |       |       |       |       |
CATATCGCCG CGGCCGCCG CCACCAACAG GCGGTCTGG CGGGGCTAC GCGGCTACG CGCCGACGG CGCTGCAGTA CGCTGCCCG CCCAGTCGC
201    211    221    231    241    251    261    271    281    291
|      |       |       |       |       |       |       |       |
CGGCCACGCG CGCCCGGGC AGGCCCGGC AGGCCCGGT CGCGCGGTG CGCGGTACCC TCGECCGCG GCGCCGGCA CGTGTCACG TCGGTCTGT TGAGACGAG
301    311    321    331    341    351    361    371    381    391
|      |       |       |       |       |       |       |       |
CACCGGCTAC TCCCGCTAC TCTCTCTAC ATGCTCCAGA TCGCTGGCG CCATCGGGT GGACTCGAG CACGTTTG CTTCGGTAA GGTTTTCT
401    411    421    431    441    451    461    471    481    491
|      |       |       |       |       |       |       |       |
AGAGGGATA GTAGCTTAC CACGGCTCG CGCCAGGCGC AGCGGGAGCC GTCGAACCT ACCTGAACG GGGTCACCTT GATGTRCCG TCCTAGCCGT
501    511    521    531    541    551    561    571    581    591
|      |       |       |       |       |       |       |       |
CCCACAGCAT CCTAGGTTAG GTTGACCGT CGGGGATCG GCGTGTCCAC ACTCTAAGCT TTCTCTACA GCGGGCGTCG TAGTAAAGAC GCTCTCTACG
601    611    621    631    641    651    661    671    681    691
|      |       |       |       |       |       |       |       |
CTCTGTAGT CGGGATAATA GTATTCCTA ACTGCACTAC AAATCTATGT ATTGGCTA GTAGGTGT CGAGTCACC TTGTTCACTCA TGTACTCTACG
701    711    721    731    741    751    761    771    781    791
|      |       |       |       |       |       |       |       |
TGTTACAGT CGGGATAATA GTATTCCTA ACTGCACTAC AAATCTATGT ATTGGCTA GTAGGTGT CGAGTCACC TTGTTCACTCA TGTACTCTACG
801    811    821    831    841    851    861    871    881    891
|      |       |       |       |       |       |       |       |
ATTGGTACAC GGACCCGGCG TGTTCGGACG GACGTATGAA TATGTCCTA GTAAACGATG TACCCACTT GGAAACGTA TCCCATGCAG TAAGGATAG
901    911    921    931    941    951    961    971    981    991
|      |       |       |       |       |       |       |       |
TCCGTCCATT ATTAACCTAG GAAACACTCAT AACAAATCGA AATCTGGAA GTTTTCGA CACCACTT ACAGTCCTT TGTCAGAAC ATCATTCGG
1001   1011   1021   1031   1041   1051   1061   1071   1081   1091
|      |       |       |       |       |       |       |       |
TTTACTCTAG ACTGAAATT AGGAGACGT AAAGAGTTTC TGTGTTCTC ATGAATCTT CCATTATAGC TCCATCCAGT TTCTAGATT CTATATATGC

```

Fig 5.1

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1101 1111 1121 1131 1141 1151 1161 1171 1181 1191
 TTTGGATC GACCCGTAC CACCGTACA TGGAACTCC GAAATATA GCTGGGTTTG ACTACCAGT CCCATTGGT GGCAGAGTC TTAAAAGTC
 1201 1211 1221 1231 1241 1251 1261 1271 1281 1291
 TTGACAAA AATGCAAGTT TCTCTGAT AACCTGACTT GGACTAGTT CGTGGACATC GTACATGTC ATTATTGGT CATGGTAAC GGTTACATGA
 1301 1311 1321 1331 1341 1351 1361 1371 1381 1391
 CCGTCATTA TCTTTAAC AATCATAGA TACACTTG CTAAGTCG AAATGATA AGCTTAATT TTACATGTC TCCPAACGTA ATTGECGTTT
 1401 1411 1421 1431 1441 1451 1461 1471 1481 1491
 TACTTAGCCA TGGCTGCTT ACARAACTT TACGATAC AGGATTCTC TCTAGTATC TTCTAAAGTA TAGATTTACG GGCTAACCGG CGACATTAGC
 1501 1511 1521 1531 1541 1551 1561 1571 1581 1591
 GCACATATA GCGGGGCAA GCTGTATCPA TCGCTGATA ATGTCGTAA TAAGCTTCT GCTTCCTG GAAATACG ACAGGAACT TAGAGACTG
 1601 1611 1621 1631 1641 1651 1661 1671 1681 1691
 TCCAGTTC TTTCACCAA AGACTTGAC CTAAGCAACCA AGCGCTGTC ACTCTCACT TATATAAA TATATAAA AACPTCGTAT GTTAATCCRT PILL
 1701 1711 1721 1731 1741 1751 1761 1771 1781 1791
 ATTACCAT ACCACGCC CTTCCTTC ACCACGTTCT ATCTAAAGGA TACCTGTAAG GGTATGTC GTTATGTC GATAAGGG CGCTGTAGCC AAGACGTC
 1801 1811 1821 1831 1841 1851 1861 1871 1881 1891
 ATGGGCTT CCACCAAC GTCACACTC GGAGCTCTAG AGCTTGCTGA TCCCACTACT GTGTTCCTC CATTACCTGT AACCTTTGAA
 1901 1911 1921 1931 1941 1951 1961 1971 1981 1991
 GGGCACAG TGTAGCTT TCCAACTCT TAATAGGTC GTAAAGTCG TCAATCCCA TGCACTAGG TGATGCTA CCAGTTCCC
 2001 2011 2021 2031 2041 2051 2061 2071 2081 2091
 CAACCTGGG AGGAACCTCC TGTAGCTT CCACAAACG TCTCTAAAGCA TCTCTCTGTA CTGTCTTGT TCATTGCTA TGTCTCCCA
 2101 2111 2121 2131 2141 2151 2161 2171 2181 2191
 AAACCTGTGA TTTCGGATC CGTTGAGT AACCTGTC CTTCCTCTC GCTCTCTC TTCTACTCTC TCTATTGTC TGTAATGTCG ATAGCCAG
 2201 2211 2221 2231 2241 2251 2261 2271 2281 2291
 TAATGCCAC TGTTCCTGAG ATTTCGTGA ATATTAATCG GGGTTTATT ATTGACATCG TGTGCTTAC TCTACGTTCT TCTACGTT CTACGAACTT
 2301 2311 2321 2331 2341 2351 2361 2371 2381 2391
 GCTTAGAAA TTGAGGAACTT GAATAAGCC TCGAAATCC ACCAAAGCA ATGAACTACA GTGGAACATCG TCACTGTC TAGTATCC

Fig 5.2

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2401 2411 2421 2431 2441 2151 2461 2471 2481 2491
 ATCAGCACT CCTCCCTGT GACGCTCCAC TGTGTTAAAG TTAACTCTC TOTALTAYTTT GAGGTAAAM AAATATCAA CATTGAACCG AGTCACAT
 2501 2511 2521 2531 2541 2551 2561 2571 2581 2591
 TTTGTATT TATGCCAGA CGTATAGAC AAACCATCCA TAATGAAATC CGGAAACGGAT ACTACAATAAT TAGTATCGAA CTTATCAAAC GAATGTTTA
 2601 2611 2621 2631 2641 2651 2661 2671 2681 2691
 CTCGCGTG TGCTTCGTAT TTTTGTGAT ACAGATCTT CAGTATGTT ACAAAATCTA TAGCTCCPCT AGGATCGGGC ATGCTAACGT CTGGAATACT
 2701 2711 2721 2731 2741 2751 2761 2771 2781 2791
 ATTTTTAAC TGTCTGATTA CGAACGTGA ATTGGTGA TCTCTCCACC AGTACATTGG CAATCTCTAC ACAGAGACT TATTTTATA ATATTGCT
 2801 2811 2821 2831 2841 2851 2861 2871 2881 2891
 ACAGAAGCTA TATGCCACAT GAAATTATG CAAATTAAT CCATCTGTAT GTTTAAACC GGTTTACTAG TCCTGTAGA GTAGCTATCC ATGATAGTGT
 2901 2911 2921 2931 2941 2951 2961 2971 2981 2991
 TTCCCTCGCC AATACTCTG GACATTATTC TGTTAACAC CATTACAA AACCTCTTA CGGTCTGTAT GTTGTCAAA GTTCTTATGT TTGAGCACT
 3001 3011 3021 3031 3041 3051 3061 3071 3081 3091
 TTGGTAAAC CATATGTAT TTTCAAATT ACTTGTAA ACTGTTAA ACTCTTAA AAATGTTAA CTTGAAAGCC GCTTGGGTTA
 3101 3111 3121 3131 3141 3151 3161 3171 3181 3191
 TCTCCGTTA TAAccccccc TCTCTTGTAT TATTCGGTA ACAAACTCT TACAACTTT CTAGTCTTC TAGTTATCA TAGCTTAA TTCAAAAGGCT
 3201 3211 3221 3231 3241 3251 3261 3271 3281 3291
 TTGTCGAGT AGGGTTATA AACGTTATA AGTCAGTCAG TAGCTCTCC ATTATTAAT AACATTTAC AGACGGCAC ACAGGGTTA TCTATATATCT
 3301 3311 3321 3331 3341 3351 3361 3371 3381 3391
 GCCTATCC GTCTGACAT CTATTTCT GTTGTAGCT AGAGAGCTT TACGTAGCTT CTCCAACTGT CTTCTAGTC TGTCTAACCG GTTACCTGTT
 3401 3411 3421 3431 3441 3451 3461 3471 3481 3491
 TCTCTGAGC AACGAGTTAT AGCTGTATAA CTTGTATAA AGCTTAGAG GCGCTCTCC ACACCTCTT TACCTGGAGC TCCAGCCGG TACACTCCGT
 3501 3511 3521 3531 3541 3551 3561 3571 3581 3591
 TGGTGAATT GCTCTATCA TCAAGCTGAG TCAATAGGTT TCTCTGCTCA TTTTCATCCA TATTGACTCC AGCAACACA ACCAGTAGA TCTTCTCTCA

Fig 5.3

3601 | 3611 | 3621 | 3631 | 3641 | 3651 | 3661 | 3671 | 3681 | 3691
 TTTAAGTAT TGTATTAGA AAGGGCAA CCTCTGCC CTGATTCGC GCGAACAC GGTGACAG CGGAAGTCG CTCGGGGCG TGAAGATC
 3701 | 3711 | 3721 | 3731 | 3741 | 3751 | 3761 | 3771 | 3781 | 3791
 GRCCGGCAC GCCTCCACGC TCGCGAACG CCCAGGGAG ACGGGTGCGT GCGAAGGAA CCCGAATCC GAGGGCCCA CGCTCGCCG CTTGAAGAGC
 3801 | 3811 | 3821 | 3831 | 3841 | 3851 | 3861 | 3871 | 3881 | 3891
 ACCCAGGCC ACTTCTTGGG CACGTGTAAG GCCTCTGAG TGCGGTGAA CACGGCCGG TCCACCGGG GGCCGGCG CGTACGGG AACTCCAGCG
 3901 | 3911 | 3921 | 3931 | 3941 | 3951 | 3961 | 3971 | 3981 | 3991
 CGCGGTCCC CGCGTGAAAC TCGGGGATGT TGTCTGTT CTCGTAGCG GCCCAGAGT GCAGGCCAC GAACATGGG CGCCGCCCG CGAGGGCCAC
 4001 | 4011 | 4021 | 4031 | 4041 | 4051
 GCAGAGCGCG GACACCGCGT CCATCTTTA TGTGCAAGAT TATTCCTGG C
 ←E2L PE2L

Fig 5.4

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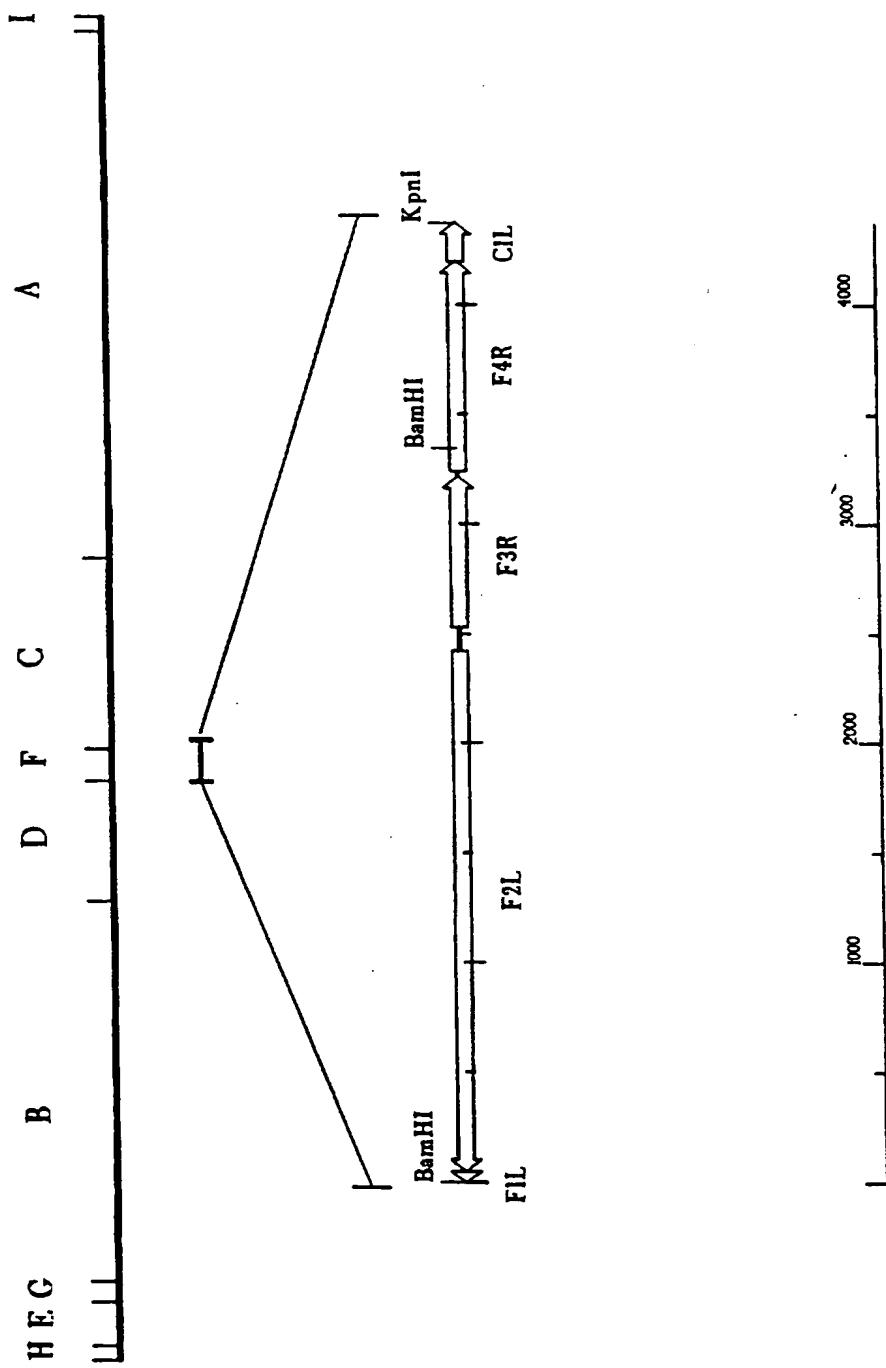


FIGURE 6. BamHI Map of Orf Virus NZ-2 showing the location and orientation of the reading frames for the putative genes F1L, F2L, F3R, F4R (topoisomerase) and C1L.

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Figure 7. Nucleotide sequence of BamHI F and part of BamHI C from orf virus strain NZ-2 showing potential insertion sites.

1	11	21	31	41	51	61	71	81	91
←FIL ***** PP1L *****									
GGATCCATT ACTTAA ATTACATA TACACTCTT TTATCGGGC TATGGATTTCG CCTTGAACCGA GCACAGTGC CCCGCCAGGA									
101	111	121	131	141	151	161	171	181	191
ACAGGAGAA GCGGAACTCC AGGTCCACCC GGTTCAAGAGA GTTCGTAAG TACACAGAGA CGTGGCTGTC CGGGAAAGAC CTGGCCGGAA ACATGGTGTAA									
201	211	221	231	241	251	261	271	281	291
CCCGTGACC TTGTGCCGA CCTGCTCGC GCGAGGAGC GTCTCGTGA AGGGTACGG GTCGCTGAAG CGGAACACGT ACATGGCCGG GTTGGCTAG									
301	311	321	331	341	351	361	371	381	391
TAGTACTCA TGGTGTGT GACGAAAGG CTGGCAGGG AGATGATGAT TTTTCTTC TGATCTCGA TCTTGATGTC GTCTCGAAG CGCTTCATGT									
401	411	421	431	441	451	461	471	481	491
TGTTAGGGT GTGTTGTCACGACGGGGATGA GCACGGGGAA GTCCGACATG ATGTCCTGGA ACTTCGGGG CGCTCTGGG CTCTGGGG GCGCTCCGC									
501	511	521	531	541	551	561	571	581	591
GGGGCGCCG ACACCGCCG CCTAGGGCCG GGGGGGGC GCATGGGGC CGCCCCAACG CGTGGCAAG CGAAAAGTC CACGGGCCA									
601	611	621	631	641	651	661	671	681	691
GGCTCGCCG CGACGCCGTA CGACGCCACC AGCTAGTTCG GCGCTGGCT GGGGGGGC ATGGTTCA GCCTGGSCAG CTTCGGGACCC AGCGGGGGT									
701	711	721	731	741	751	761	771	781	791
AGTCCGCTC CAGCTCCCTCG GGCATGATGCC AGGTGTACAC CTGGTGAGC ACCATCACCG TGTCGAAGTC CTCCCTGGG AGACGGCGC TCTTCACGAG									
801	811	821	831	841	851	861	871	881	891
GAAGCTGGC ACAGCCGTC CGATGAGAG CGCGCACTG GACTCGTGA CCTCGACGCT GGCTCCCTTG GTCCTCTTG AGCTCCCGA GCCCATGAC									
901	911	921	931	941	951	961	971	981	991
GAGACAGGA AGTCCGCTC GCTGTGAGC ACCATGACCA CGCGCACGAT GAAGCTGAGG TTCGGGACTT TCCTGGACTG GAACAGCTCG GTGGCCSACG									
1001	1011	1021	1031	1041	1051	1061	1071	1081	1091
CGTGCAGTC GAGCAGGTC GCGAGGAGC GCAGGAGAA CACGGGGCC TGGATCTGG CCTCGAAGGC ACCTTCGTAC TCCCTGGGG GCGGGTTGAT									
1101	1111	1121	1131	1141	1151	1161	1171	1181	1191
CGGATGAGG AAGTTCAAGA TGACCCGGTT GATGTTAC GTCACGGC AGGTCTGGT CTCATGATG GTGTCGAAGG ACATCACGAT GTTGAAGATA									

Fig 7.1

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1201	1211	1221	1231	1241	1251	1261	1271	1281	1291
	AAGGGCTGGC	TGTCGGAGA	GTAGCTTAG	GGCTCTCTGA	CCAAGAGGA	CTTGTGGTC	GCGGCCACCA	CCACGCCCGC	GACGGCTGGG
1301	1311	1321	1331	1341	1351	1361	1371	1381	1391
	TCTTCAGTC	CGGGATGTC	ATGCCGGAGA	TGCGGGAGTA	GGCCATCG	TCCTCAAGT	ACAGAACTC	CTCCACGAAC	TGTTGATCT
1401	1411	1421	1431	1441	1451	1461	1471	1481	1491
	GTCACGCTCC	ACGGCCATG	CGACCGGAGA	CCGGATCTGG	TGCTCCAGG	GGGGGACTC	GAAGGCAC	CCCTGGCCCC	AGCCCCGGG
1501	1511	1521	1531	1541	1551	1561	1571	1581	1591
	ACCAAGGGCG	TGGCCGAGG	CGGGGGAGC	TGGGGGAGC	GGGGGTGAG	CAGGGGGCG	AAGGGTCCC	AGAGGTGCG	GCTEGAGGG
1601	1611	1621	1631	1641	1651	1661	1671	1681	1691
	TGTAACGGAG	CCGGGGCGG	TCCGGGGCA	TGGGTCAC	GAAGGGGG	CCCCATCCG	CGACCGGG	CTCTCTCC	GAAMGTTG
1701	1711	1721	1731	1741	1751	1761	1771	1781	1791
	CTTCCTCGTC	GGCGCGAGA	ACACCTTCTT	CACGTCAGG	AAGTCGGGA	TCACTATGG	GAACGGGG	CTCTCGAGCT	CACGTAGCAA
1801	1811	1821	1831	1841	1851	1861	1871	1881	1891
	AGGTGAGCT	TGCGGGCGA	GACGGGATG	CCATGTGCC	GACAGGTA	CGGGAATCTG	AGTACTCT	TGAGAAGG	GATCGGTCC
1901	1911	1921	1931	1941	1951	1961	1971	1981	1991
	AGAGGTACTG	CAGCATGTC	CCCATGTTGA	AGGGATCTC	GGGACGGG	GGCTCCCGG	CGTCGTGAA	GGGGTGGG	AGATCGCTGC
2001	2011	2021	2031	2041	2051	2061	2071	2081	2091
	GACCAAGGCT	TGCCGGTGG	CTGCGCTG	CACCAAGC	TAAACGCC	GTCGGGGAT	GACCATCTG	AGAGTGTGA	AGAACATCTC
2101	2111	2121	2131	2141	2151	2161	2171	2181	2191
	TGCGGAGCT	CGTCGGCGAT	GGCTCTCCC	ACCGAGACG	TGCGGGGG	GTCGTACGC	ACCTGTTCT	CGAACTTGTA	CCCGATGTTG
2201	2211	2221	2231	2241	2251	2261	2271	2281	2291
	AGATCAGGCT	GGCGCTGTC	GGTCGGGGT	TCTGCTCCAT	GGTCGGAA	AGCAGGGG	TGTCGTCTC	CGTGTGG	ACAGGGTGCAC
2301	2311	2321	2331	2341	2351	2361	2371	2381	2391
	CACGAAGATC	GACCTTCTC	CGGGCATCA	CTCTGGTGTG	CTCTGGTGTG	CTCTGGTGTG	CTCTGGTGTG	CTCTGGTGTG	CTCTGGTGTG

Fig 7.2

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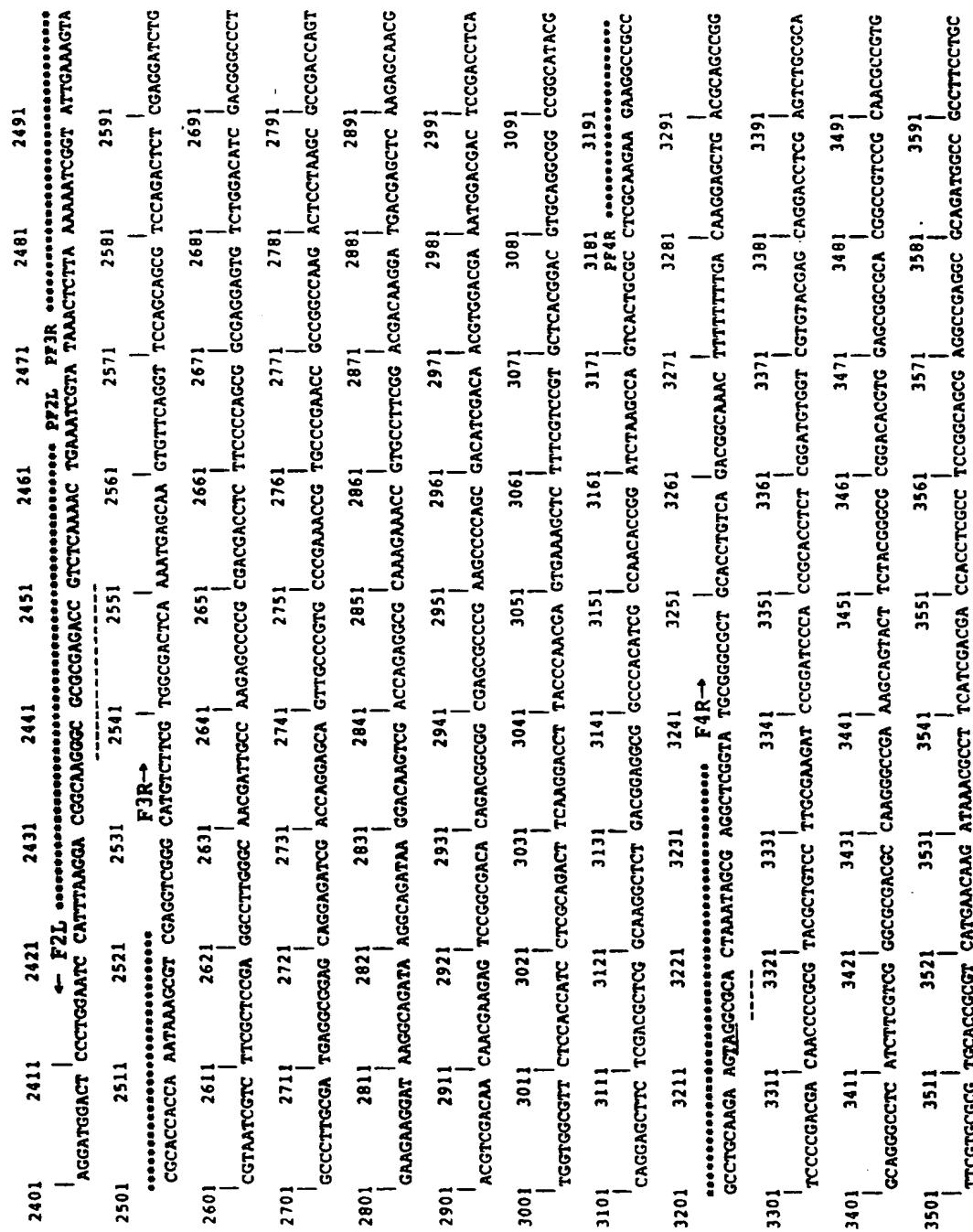


Fig 7.3

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3601 3611 3621 3631 3641 3651 3661 3671 3681 3691
 TCTTGACG GAGGTCTTC ATCCCGTGG GCAGACGG CTACGGGC GAGCGGA CGGGGCCAT GCTCACGTC CGAACAAAGC ACCTCCCGA
 3701 3711 3721 3731 3741 3751 3761 3771 3781 3791
 GGCGGGC GTGAGGACA TCCGGGCG CTTGGGCG AGGACCGAG TCCGACCA GTTGGCGT CGGAGGGGG AGGGCTCTT CGGGGCTG
 3801 3811 3821 3831 3841 3851 3861 3871 3881 3891
 CCTGGCTCT GGAACCCGGG CGGCCGAC AGGGCTGTG TGACCGCTT GAGGAGGC CGGTGTACA CCTCATGG ACCGTCGGC ATCCGGCTCA
 3901 3911 3921 3931 3941 3951 3961 3971 3981 3991
 AGGACCTGG CACTACGCC GTGAACTACA CCTCTCTGTA CAACTCTGG TCCAACGTCG GCTGCTGGA GCCTGTCCC TCCGTGAAGT CACTCATCTG
 4001 4011 4021 4031 4041 4051 4061 4071 4081 4091
 CACCTGGG CGCGAGCC CGGAGACGGG GGGCACGG CCCTCTGATCT CGGGAGGGC CTACATGGGC ACCGGGTGC TCGGCTCGT CAGGGACGGC
 4101 4111 4121 4131 4141 4151 4161 4171 4181 4191
 GGGTCTGG ACAGAGTGG CGCOACCGAC ACGCTGACG ACTCTGTGA CATCTGTGA GACTATGAA ATTACTGAA GCAGTAAAT GGATGAGGC
 4201 4211 4221 4231 4241 4251 4261 4271 4281 4291
 CTGGCGGG CGCGCCGGT CGTGACCGG CTCCGGCGC TGACCTGGC CTGCTGTC GCGAGGTGC GCGGAGCCG GCGGAGGC CGCTTCCGG
 4301 4311 4321 4331 4341 4351 4361 4371
 CGCTGACGA GTGCTCCGAC GAGGCCCTTC TGAACCTTGA GTTCGGGGC GCGGAGCTGC GTCTGGCTA CC

Fig 7.4

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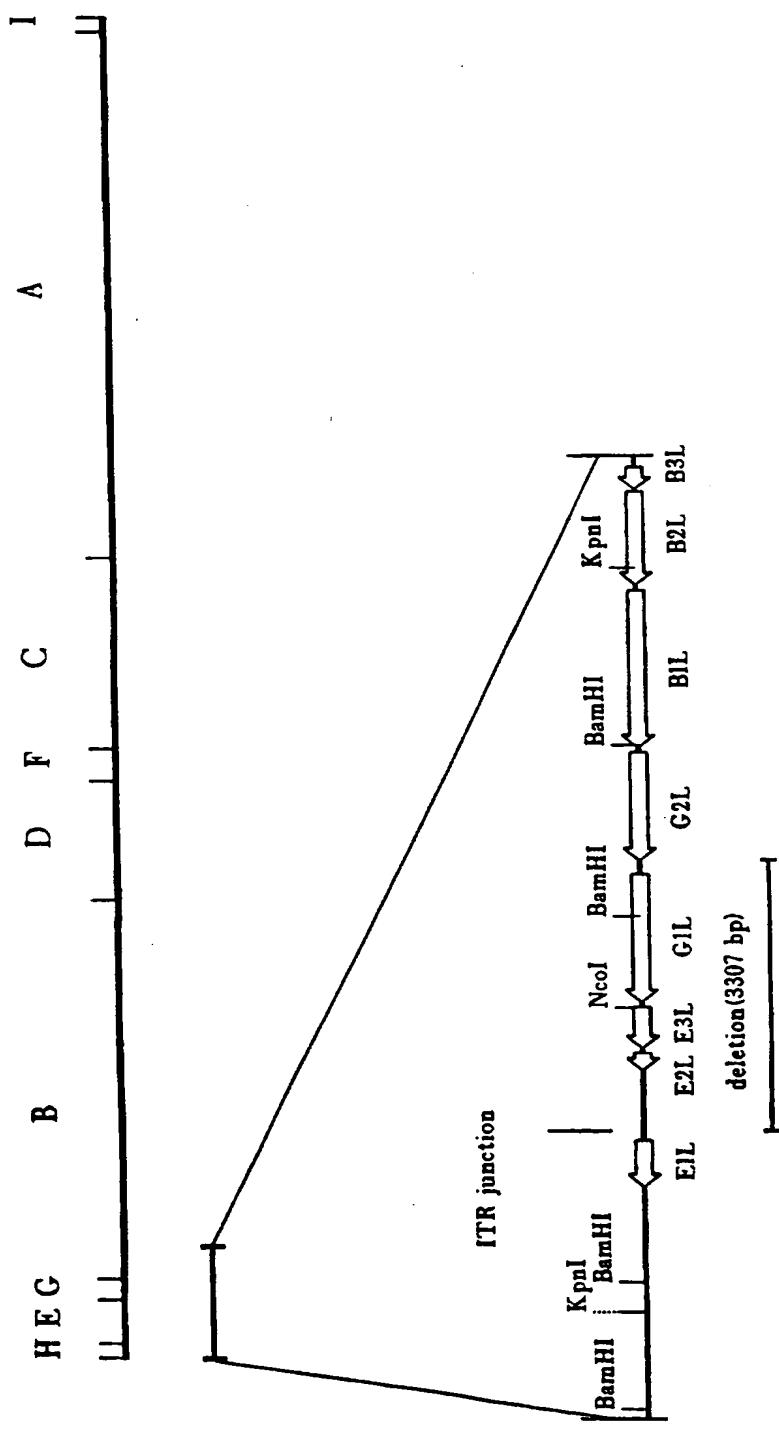


FIGURE 8. BamHI Map of Orf Virus NZ-2 showing the location and orientation of the reading frames for the putative genes E1L(ORF-3), E2L, E3L(ORF-PP), G1L, G2L, B1L, B2L, and B3L.

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Figure 9. Nucleotide sequence of part of the BamHI E and BamHI G fragments from orf virus strain NZ-2 showing potential insertion sites.

1	11	21	31	41	51	61	71	81	91
AGTAGGGG CGGGGATAA <u>AAATTA</u> TGA <u>CTGATT</u> CGCTCTAG CGAGGGAGG CCCGGGACA AGGGGGGG AttCTGGTC AATCTACTAA									
101	111	121	131	141	151	161	171	181	191
GGCGATTAC AAAACGAT GGGAGACGG GAGGGAGG GTCACAGCTC CGAGGCTC ATCCGGCA GCTGGGGG CCACTGGGC GCGGGCGG									
201	211	221	231	241	251	261	271	281	291
GCCGCCAGG CGCCGTTATA GCCCCCTATA ACCGGCACGC AGGTCTCTC GAATCTGGC CGAGGGCC GCACGTCATA GATGTCACG GTGCCGACGT									
301	311	321	331	341	351	361	371	381	391
TCAGGAGCAG CGGGCCCTTG CGAGGCGAG CGAAGTGC GTGGATCGG GGGGAGGG GTACACGG CGAGGGCTC CGGGCCCTG GCGGACAC									
401	411	421	431	441	451	461	471	481	491
CGCCATGTC CGCCCGTGC CGCCGACGCT CAGGCAGGTC ACCGGGG AGCCGTTGCG GGGACGGG CCCGCCTCA GAGGGGGC CGGAGGGC									
501	511	521	531	541	551	561	571	581	591
GGCCCGCGG GCGGAAGAG CCTGGAGAG AGGAAGCCCG AGGAGCCCG AGGAGCCCG CGGAGACAG CGGAGCGGG CGAGGACCC TGGGGGGGA GGGGTGGATG CTTCGTTGGC									
601	611	621	631	641	651	661	671	681	691
GGCTCTTGG CGCTCTGGG CGAGGAGCG GTCTGGCT GTCTGGCT GTCTGGCT GTCTGGAG GGTCTGACT CCTAGGGGT CCTTTTCAC TTTCCTCCGT									
701	ITR	711	721	731	741	751	761	771	781
GGCCCTCTGT CCTGGCAAG CCTCTGGGG CGCTCTGGG CGGGGGCCC GCTACCCCG CGGGGGCCC GGGGGCCA GGGGGGGC CAGGGCGGC									
801	811	821	831	841	851	861	871	881	891
CACCCGGAC CGCCGGCCC TAGGAGCCC GCGCCGGCC GCGCCGGCC GCGCCGGCC GCGCCGGGG CGGGGGCCC GGGGGGCC AGGAGGGC									
901	911	921	931	941	951	961	971	981	991
GCGCCGAC									
1001	1011	1021	1031	1041	1051	1061	1071	1081	1091
CGCGCCACG AGGAGGCGCA CGAGGCGGG CGGGGGCCC AGGGGGAC CAGGTGCTCC AGGAGCAGGG TGGTGAGCAA GGATTCTCGA GAACTAGGAG									

Fig 9.1

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1101 1111 1121 1131 1141 1151 1161 1171 1181 1191
 TCAATGTTGA CGACAGGAG AGACCTATA TTAGGCCGT CCTACTCAC TTTCAGATG GTCAGAAGTG TAAACCTTG AACACGGTTG AACACCCAC
 1201 1211 1221 1231 1241 1251 1261 1271 1281 1291
 TCCCGTACCG GTCCTCGCC CCAAGCAA CCACAGTGTGCT TTTCCACCA CCTGTTCCA ATCGTTCGA AAAGCTCCA TCCATTGTTG TTAGAATT
 1301 1311 1321 1331 1341 1351 1361 1371 1381 1391
 CAGATGTTC TCTAGGTTG TTAGTCCAC TGCAAGTTTC GACCATTAC GTTACTGAC ATCTGTTG TAATGTTT ATAACCAAT CATAAAATA
 1401 1411 1421 1431 1441 1451 1461 1471 1481 1491
 GTTAAATTG GTTAAAGC TAATAGTA GCAAACTT TAATGTTATA TTTCCTAA CCCTCCGTTA ACACCCAT TAACACCC ACTTAAGCTT
 1501 1511 1521 1531 1541 1551 1561 1571 1581 1591
 TTRACTACCAC CACTACCACCC TCCAAACACAC ATTCTTCT CTAAAGTCC CCAAATCCA CCTCCGTGAC TITGCACGTT TACAGGACCT CGGGGTAC
 1601 1611 1621 1631 1641 1651 ← E2L 1671 1681 1691
 TGGCGTACCC TTAGAAGT CCTACTGTCAC TTGAGATATG ATACTGCTCT TCTCAGCGA TGTTAAAGT GRTGTTAAT TAGTTTATC TACGCAACTG
 1701 1711 1721 1731 1741 1751 1761 1771 1781 1791
 TGCGAGTC TCGATAAA AGAACCTACA TTTACATT TGTATTGAT GATCPACCAC GCTGTATCGC GCCTACCCAT AGCACCCGAT CCAGTAGAAC
 1801 1811 1821 1831 1841 1851 1861 1871 1881 1891
 CAAATCCAGA GTCGCCCGG TTAGTGTGT CCACAGAT ACCCTGTA ACTGTGCGC ACGATATGGC TTCGATATT AGCTGAGTA TCCCTCTCC
 1901 1911 1921 1931 1941 1951 1961 1971 1981 1991
 CTCTTAACC TCAAGTCAC TTGTTCAAA GTTAAACAGC ACCACTCGA CGTGGCTCG GTAGTCTCG TGGATCAGC CAGGGCCAC GTCGATAAG
 2001 2011 2021 2031 2041 2051 2061 2071 2081 2091
 TGTTTGACTG CAAGGCCAGA ACGGGGTCTT ATTCTGCTT AGGGGGTTT ATCAGAGGT CAGTAATAC TAGGCAGCTG CAATGCGAAG
 2101 2111 2121 2131 2141 2151 2161 2171 2181 2191
 GGATGACACA GTCGTATGCA CTACATAGGT CTATCCGC GGCAACAGGA GATCCTCTGG CTGTTAGT GCGGTTTGG CTGAGGGAA CAACCTGAAC
 2201 2211 ← E3L 2231 2241 2251 2261 2271 2281 2291
 AGTTCCCTG TGGCAGACT CCATGGTAG GGTGGCAAGC GCGGAGCA CTAGGGGTG TACIATTAC ACTTCTCCA GAAAGATCAG GGGGGCTCA

Fig 9.2

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2301 2311 2321 2331 2341 2351 2361 2371 2381 2391
 GCGGAGGG AGCAGCGAGT CGTACGACAG GRAGCACAG ATGGAGCTCA CGATCCTGG CCGCAGGGG CACGGGCACA TGAGGGCCGC
 2401 2411 2421 2431 2441 2451 2461 2471 2481 2491
 GATCTGCTCA GCGAGGGAGA CGCGCAGGCC CARCATCGAG ATCTTCGGGA AGGGGGGT CCCGTAGATG GGGACTCTGG CGCGCGCTC CAAAGGGG
 2501 2511 2521 2531 2541 2551 2561 2571 2581 2591
 TTTCTGAGGA AGAGCGCTT CGCGCTCTCC GACGGCGCA GCACGCTCAA CAGGGCTCAA TCCGGTGGGG AGGGCACCACGC AGGGATGCTT GGGATCTCT
 2601 2611 2621 2631 2641 2651 2661 2671 2681 2691
 GCTCGGAGC CGGGATCAAGA GTCGGTATG CGCGCTGAGC GCGCGTGGC AGGATGGGG CCGCCCTCGCC GCGGAGGGCA AGGTCCTGCA CGCACAGCAG
 2701 2711 2721 2731 2741 2751 2761 2771 2781 2791
 CGGGGCTCC GAGGGCTCAA ACACGGCTCA CCATGGCTCG GATGATCA CGGGGGCGC GAGGAGCTCC GTCGGGGCCC GACGGGGGG
 2801 2811 2821 2831 2841 2851 2861 2871 2881 2891
 GTGGGGCGCA CCTTGTGCGG CGCGAGCATC TCCGAAACCG CGCATAGGCC CGAGGGCAC ATGGCTCGA GCTTCGGGCC CATGGCACC AGCGGGAGC
 2901 2911 2921 2931 2941 2951 2961 2971 2981 2991
 AGGccGGGAG CCTGAAACCC GCGGCGGATC GCGGCGGATC CTGGCGGTT TTGTTGGCA GGTCAAGTC CAAACGGGC TCGGACCGA AGTCACGAC
 3001 3011 3021 3031 3041 3051 3061 3071 3081 3091
 GCGGGCGCG CAGGCGCGT AGGTCGGCAT ATGAGCGAAC TCAACCGTC TAGGGCGGCC GGTTCCACGC CTAGGGCGT GAGGGTGGC
 3101 3111 3121 3131 3141 3151 3161 3171 3181 3191
 ACCATGGCCCT CGGAGATCTT GCGCGGGCC GCGAGGGTGT GCAGGGGTAC GCGCCCGTAC CGTCCACGA CCGACGGCTC CGGCCCCGG CGCAGCATCA
 3201 3211 3221 3231 3241 3251 3261 3271 3281 3291
 TGCCACGAG CGGGGGAG ACGCCGGG AGCACCGAG CGGGCGCAG GGCGCAAG CGTGGAGTC CGTGGCTTT GGAGGCCTT CGGCTCGAG
 3301 3311 3321 3331 3341 3351 3361 3371 3381 3391
 CAGCAGCCGC AGGAGGTCTT CGGGATCCA CTGGCTCTG GCGTACAGT GCAGGGGGT TACGGCTAG GTGTTGGCT CGTTCACGG CGGCCCCGG
 3401 3411 3421 3431 3441 3451 3461 3471 3481 3491
 TCAAGGAGCA GCGGGCGAC CTGGAGCTCG GGGCGTGGG CGGGAGAGA AGGGGGAGA GAGGGAGAGA CGCTGTCGCA GACAACGAGG CTGGCGTCC

Fig 9.3

3501 3511 3521 3531 3541 3551 3561 3571 3581 3591
 AGACCACTC CGGCGCGAC TCCAGCATTA GGGGACAC CTCGGACAC AccCGGCGT ACGGCCGTGAA GGGGTGCAGC GGCGCGGC CGCAGGAGTC
 3601 3611 3621 3631 3641 3651 3661 3671 3681 3691
 CTGGCTTC ACCTCCGAC CGGCGTCCAG CAGGACCGC ACGATCGCG CGCACGCTCG CGACGCTGCG GAGGTGCGG AGAGTCACCC AGAGTGAGG CGGGGTGCG
 3701 3711 3721 3731 3741 3751 3761 3771 3781 3791
 CGGCGCGC CGGGGAAGTT CACCGTGGCG TCGGGTGGCTA CGAGGCGGG GACCGTTCG AGGTCCACCT GCCCGGACTC CAGGTAGCGG AAGAGGAGT
 3801 3811 3821 3831 -G1L 3841 3851 3861 3871 3881 3891
 CGCGCGCG GACCACGAGG GACTCCGGG AGAGATGGC GGCCTTACA ATATTAAA TCTTTTCA CTCATCTTA TGCCGCTGAA CGCGCAATAA
 3901 3911 3921 3931 3941 3951 3961 3971 3981 3991
 GGTTAGAGT AAAAAGTC TACAGAAGG TACAGAAGC GGGGGGGG GGGGGGAC GGGCTGGGT GCTGCCAGT GAATTGGCT
 4001 4011

↓ endpoint of deletion

CTACAGGGG AccGGCTCGC

Fig 9.4

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FIGURE 10. Orf Virus Transcriptional Promoters.

Early Promoters

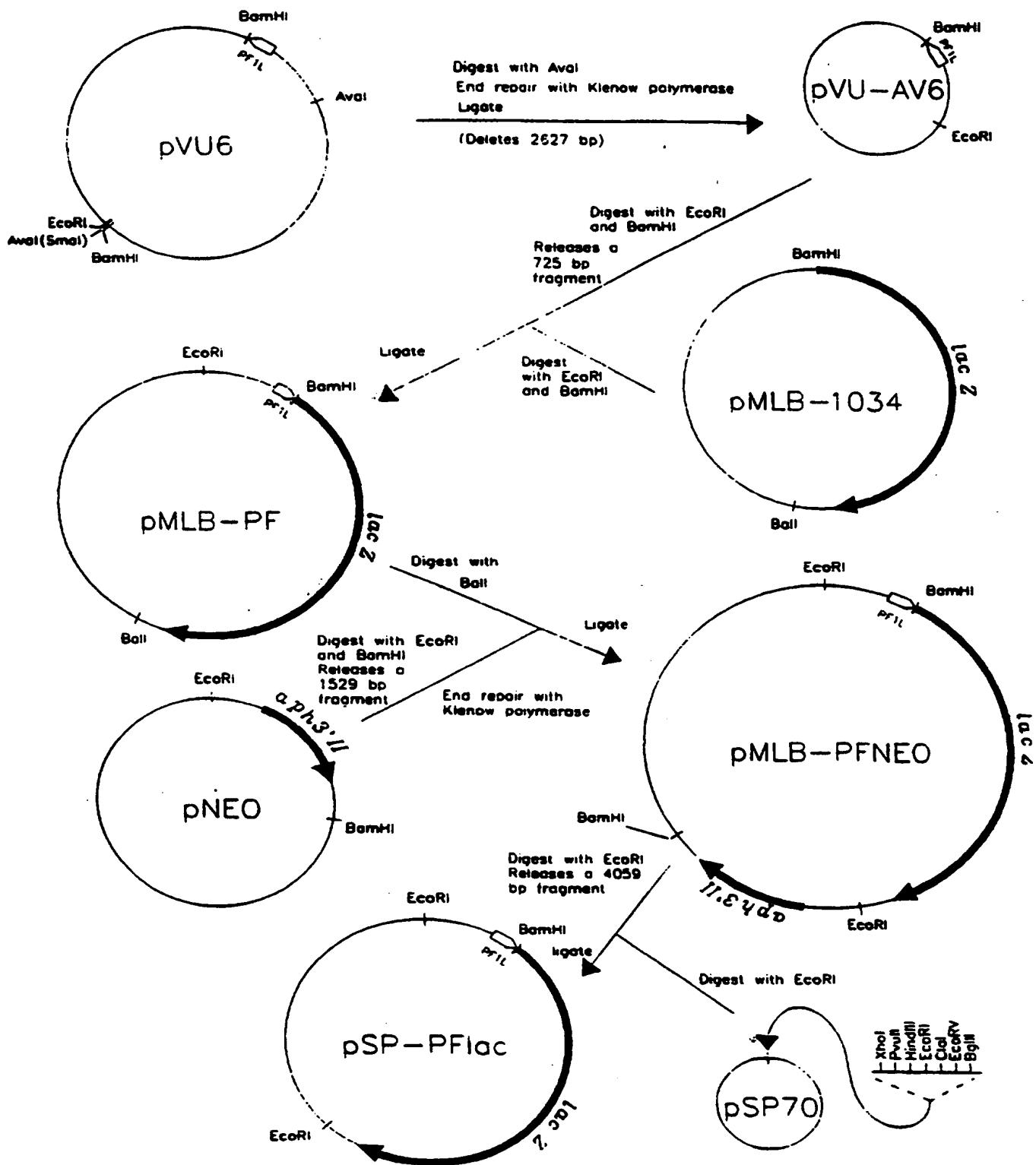
E3L (ORF-PP)	GAAAGTGTAAATTGTACACCCGTAGTCGATCGG
E2L (ORF-1)	AAAATTGTAAAATGTAGCTTCTTTATTGAGA
E1L (ORF3)	GCAAAGTAAAAAGGACCGCCTAGCAGTCGAGAC
G1L	GATGAGTGAAAAAAAGATTCAATATTGTAAACG
G2L	AATAACTGATAAAATATGTTTTGGTTGGT
B1L	ATAAATTAAAATTAAAGCGCGAGGCTCGAACGC
B3L	AATTATTGAAAATGTAGGCGCGATAAACACACGT

Late Promoters

F1L	GCCGCCATAAAAGAGTTGTATATGATTAATTAA <u>TAAATGGATC</u>
F2L	TTTCAGTTTGAGACGGTCTCGCGCCCTTGCCT <u>AAATGGATT</u>
F3R	ACTCTTAAAAATCGGTATTGAAAGTACGCACCACCA <u>AAATAGCGTCG</u>
F4R	CGCAAGAAGAAGGCCGCCCTGCAAGAAGTAGGCGCA <u>CTAAATAGCGA</u>
B2L	AAGACTTCCCTGAAGCCCTATTATTTGTGAGATA <u>AAATGTGGC</u>
HE2L	GGAGCTGCGAGCTCCGCGCCA <u>ACGAATAATTCTGCACATAAAAGATG</u>
H12L	ATATTAGATAACCGCTGTGTTGCCGT <u>CTGTAATTATTAATTATAAATG</u>
H11L	GTAATAAGGATTAACATACGAAGTTGGATA <u>ATTATTAATTATAAATG</u>
C1R	TTCGTGGACATCGTCGTGGACTATGTA <u>AAATAACTCTGAGCAGGTAAATG</u>

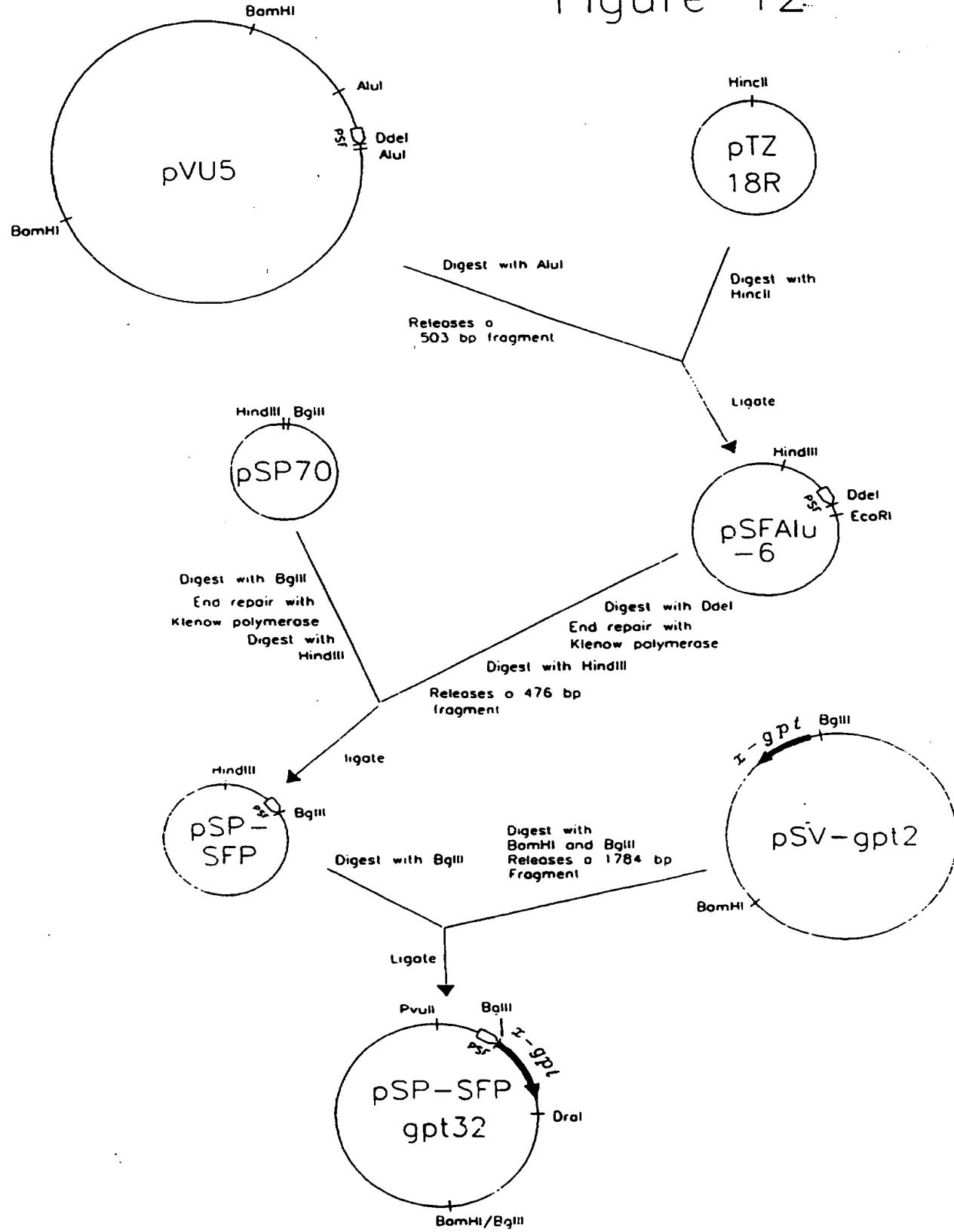
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Figure 11



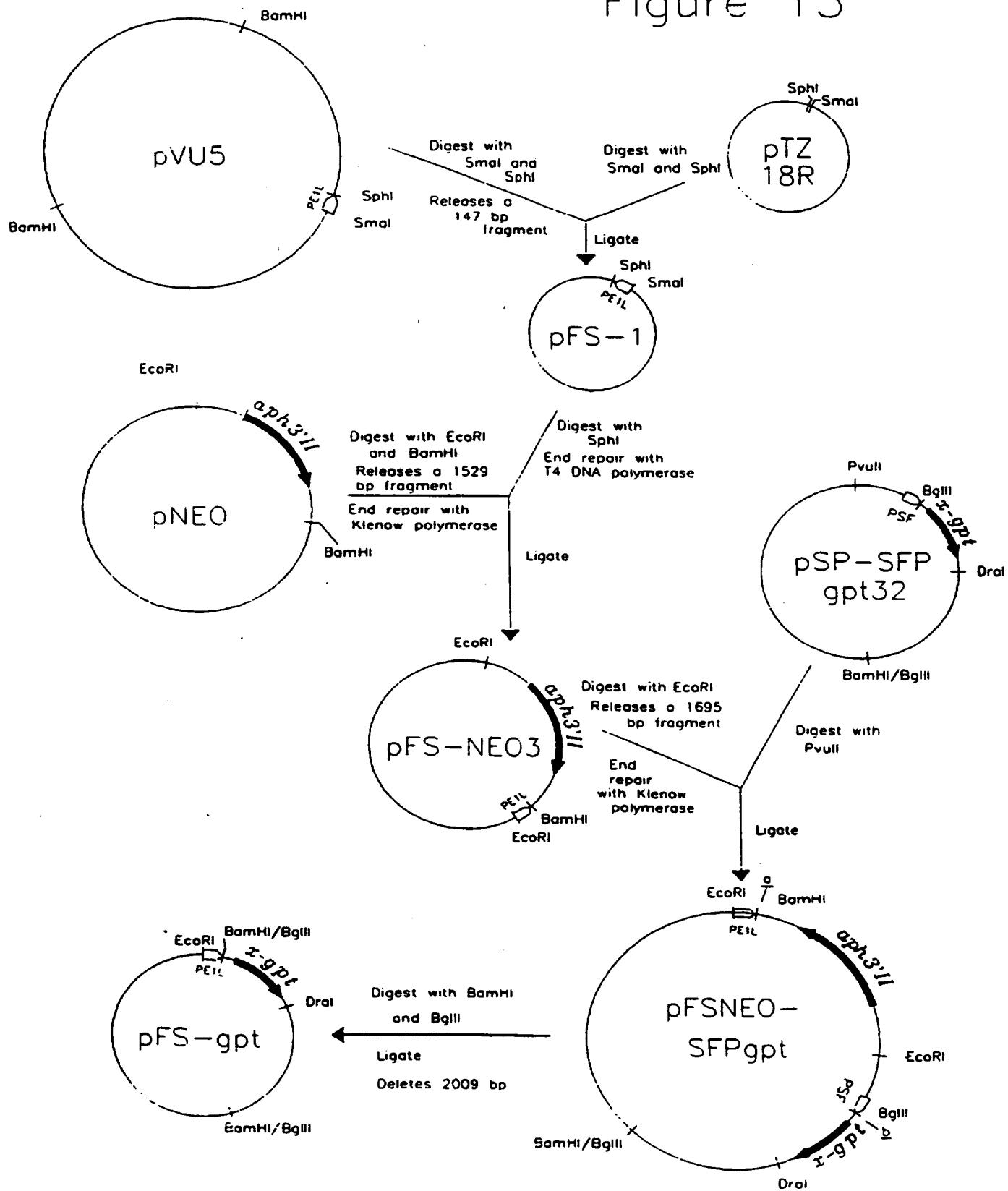
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Figure 12



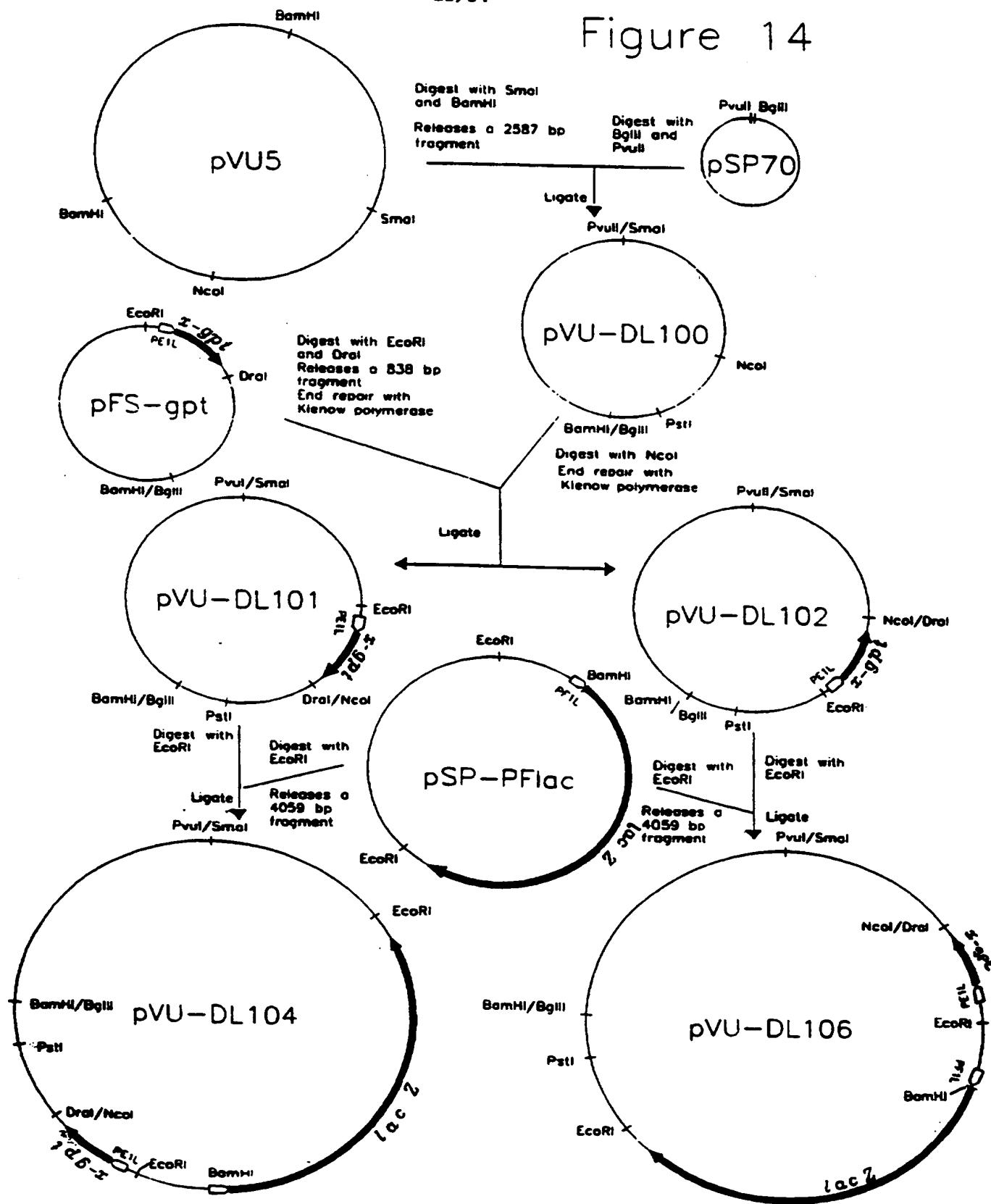
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Figure 13



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Figure 14



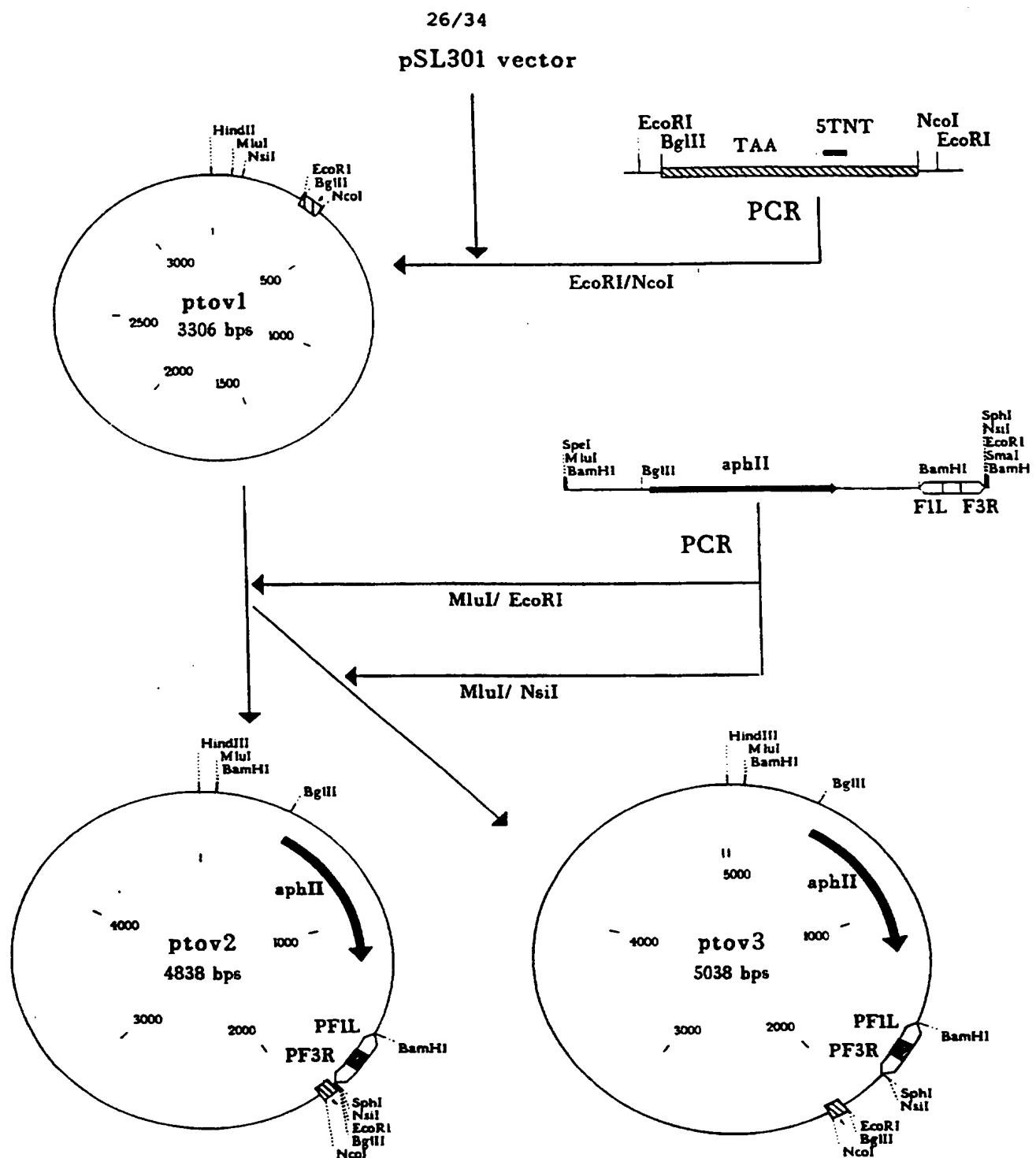


Figure 15. PCR amplification steps involved in the construction of ptov2 and ptov3.

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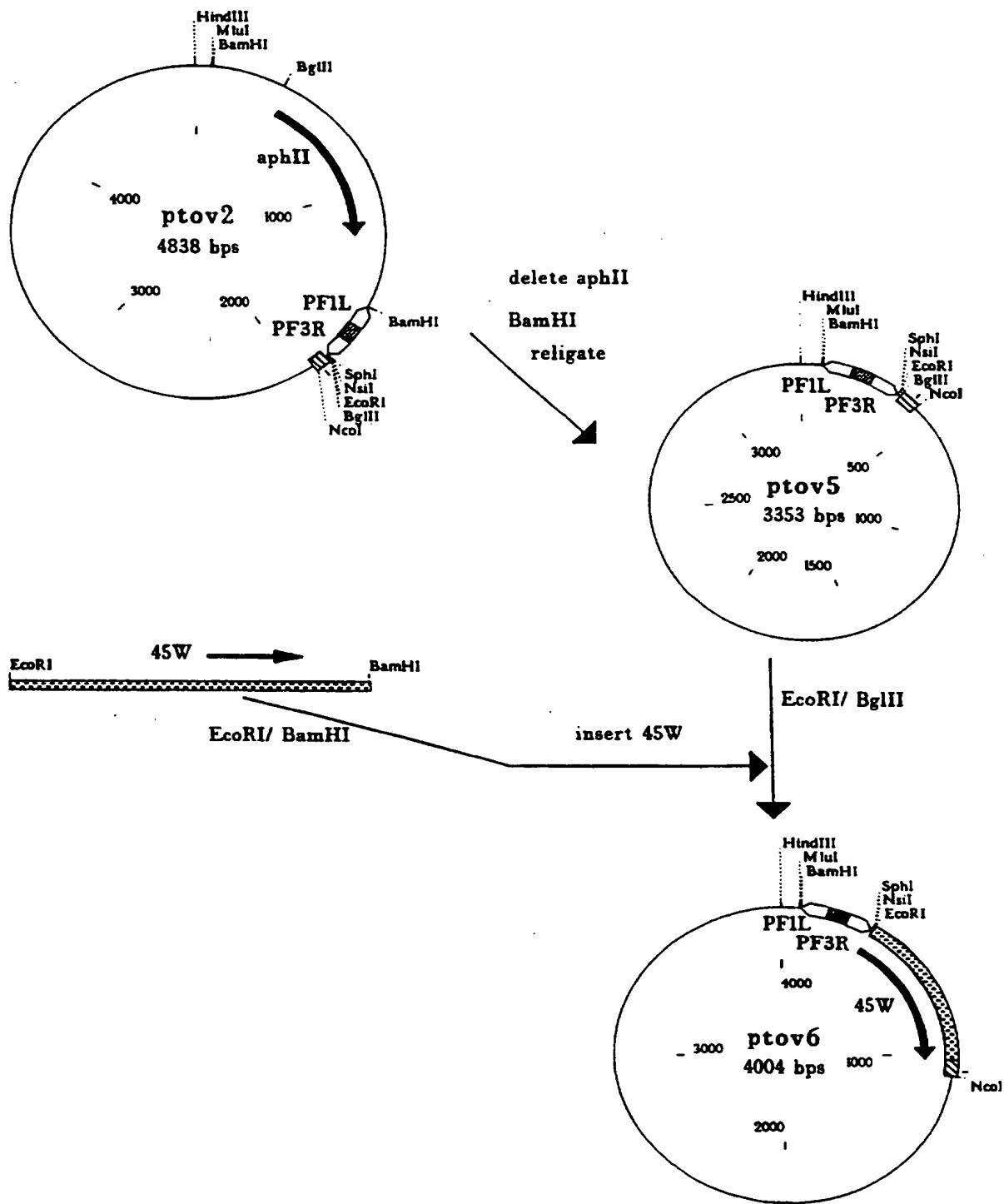


Figure 16. Cloning of the *T. ovis* 45W antigen into ptov2.

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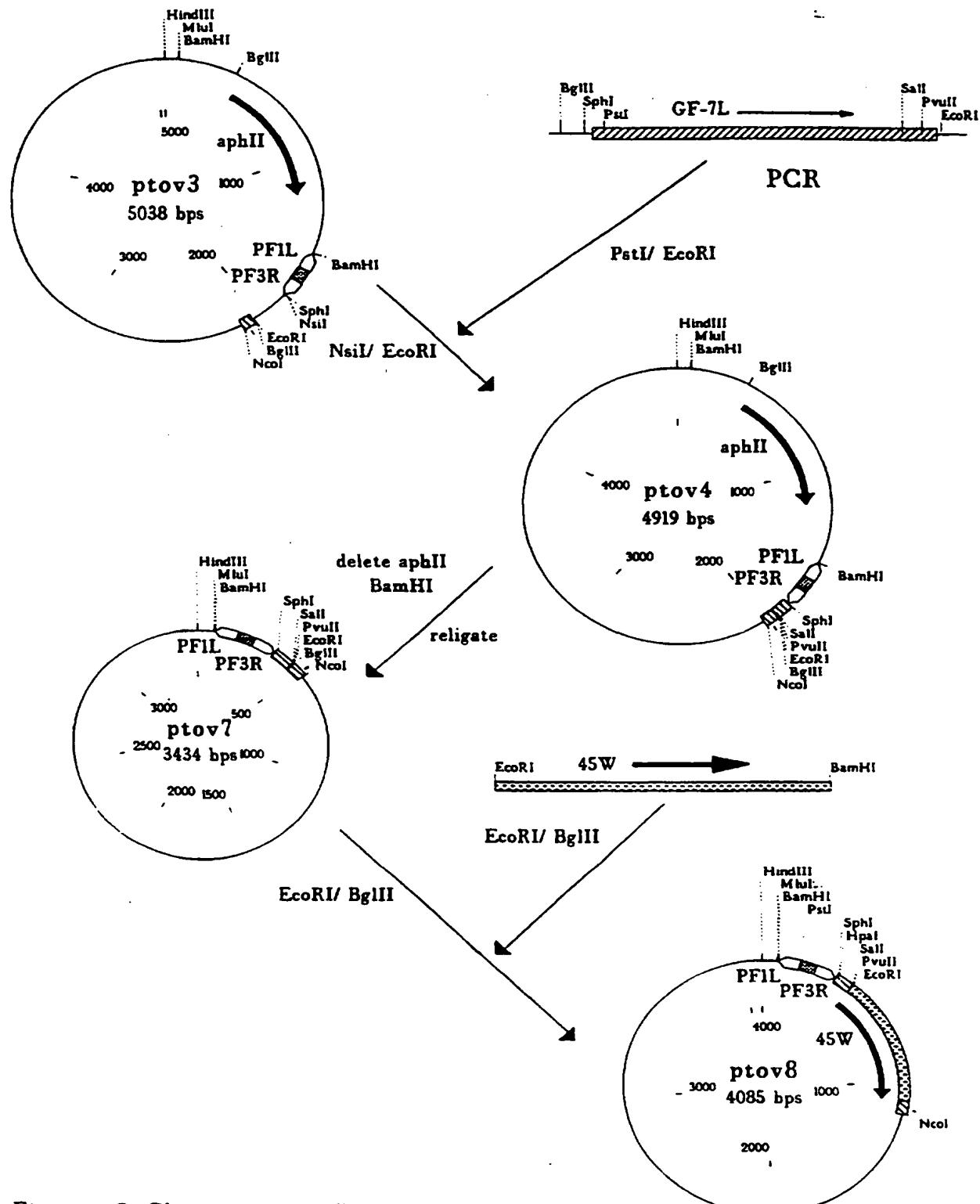


Figure 17. Cloning of the *T. ovis* 45W antigen into ptov3.

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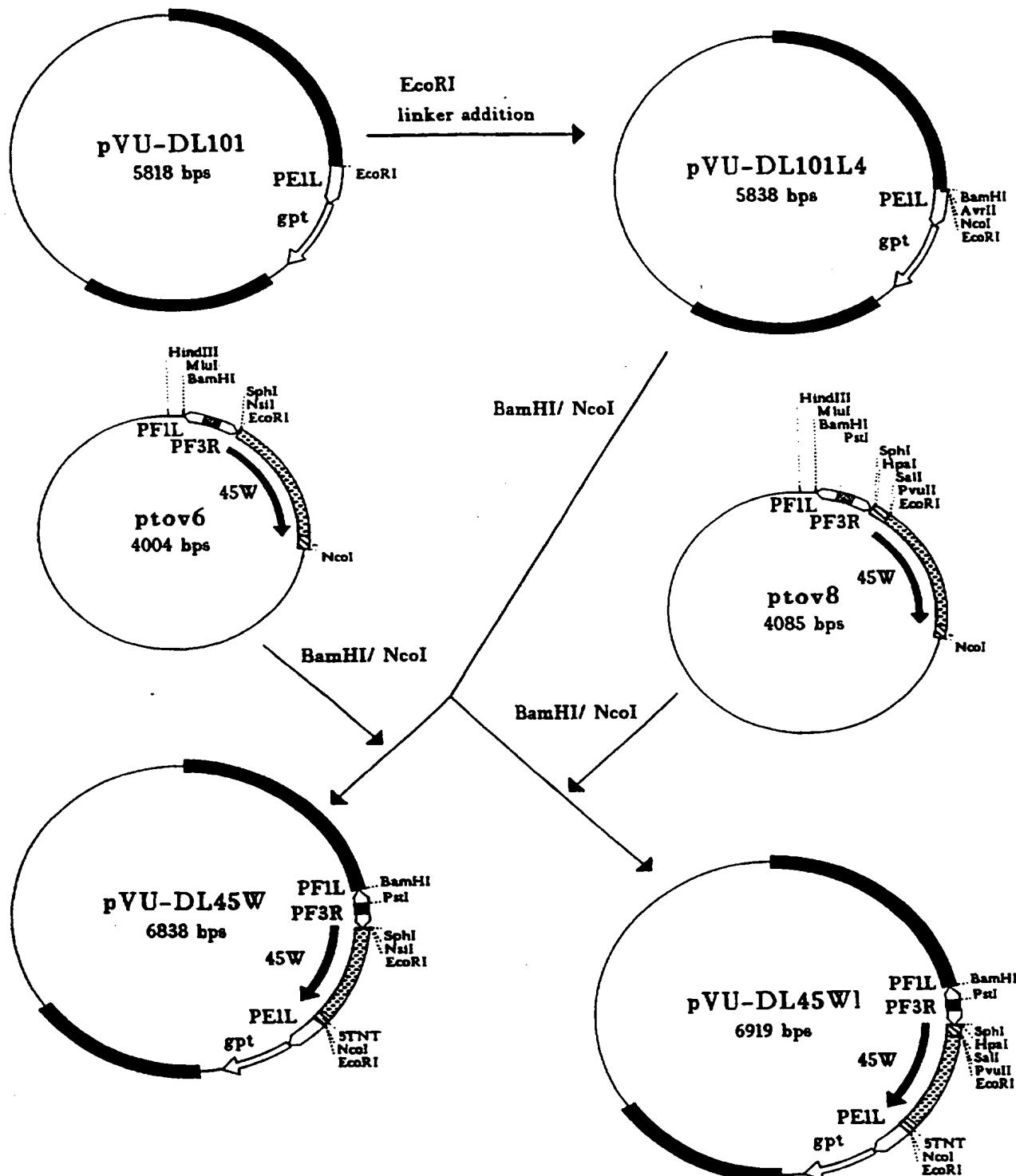


Figure 18. Insertion of the *T. ovis* 45W antigen into pVU-DL101.

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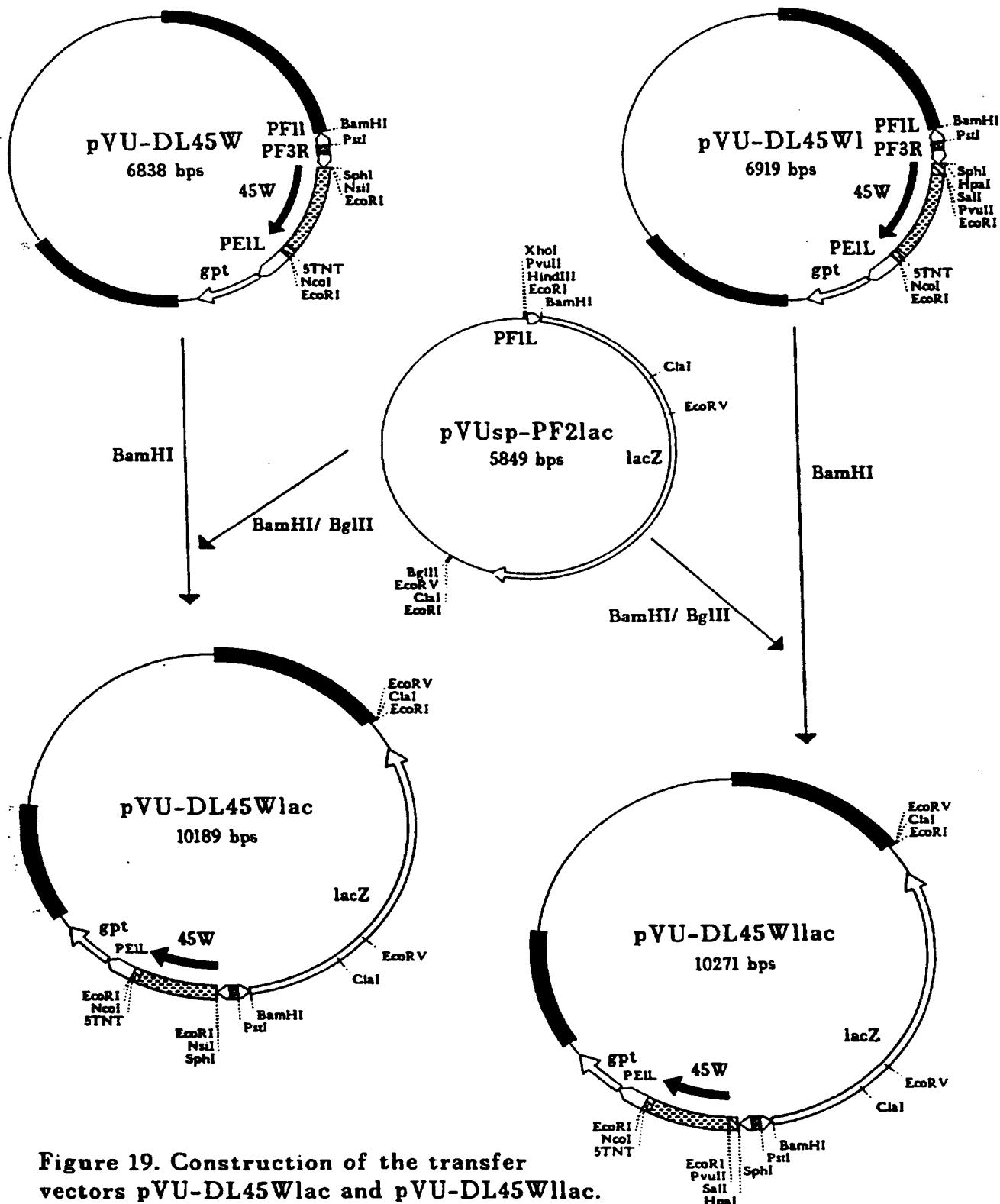


Figure 19. Construction of the transfer vectors pVU-DL45Wlac and pVU-DL45Wllac.

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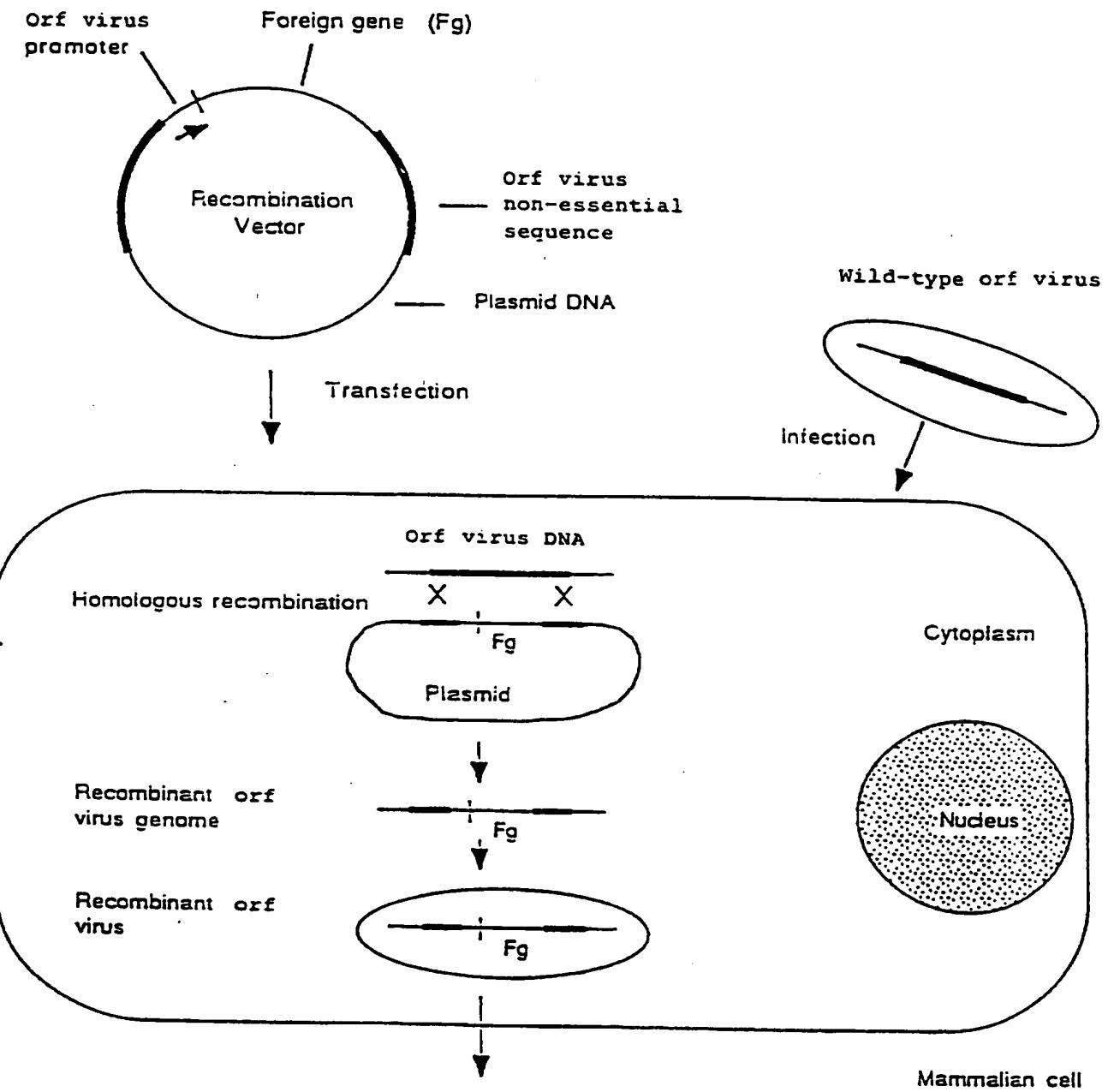


Figure 20. A strategy for the generation of recombinant orf virus.

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zxs-1 GAT CCC GCT CGA GAA CTT CAA
XbaI

zxs-2 GTC AGA TCT ATG CAT AAA AAT TTC GCA TCA GTC GAG ATA
BglII *NsiI* *ApoI*

zxs-3 GAC ATG CAT CAG TGC CAT GGA ATT CTC GCG ACT TTC TAG C
NsiI *NcoI*
EcoRI

zxs-4 GAC GGA TCC GTA TAA TGG AAA GAT TC
BamHI

Figure 21A. Primers used for the amplification of orf virus sequences used to create the transfer vectors pTvec1 and pTvec50.

1	11	21	31	41	
<u>GACTGATGCG</u> AAACGCGCGG CGGCGCCGCG ACTTAGCTTA TCTCGACTGA					*****

					zxs-2 primer
51	61	71	81	91	
<u>TGCGAAATT</u> TTATGCATCA <u>GTGCCATGGA</u> ATTCTCGCGA CTTTCTAGCT					*****

ApoI	NsiI	NcoI	EcoRI		zxs-3 primer
101					
<u>TCTCAGACTG</u> ATGCTAC					

Figure 21B. Sequence of modified intergenic region between the RNA polymerase subunit gene, rpo 132, and (H)11L in pTvec50, showing new created restriction sites for the restriction enzymes *ApoI*, *NsiI*, *NcoI* and *EcoRI*. The priming sites on the original OV sequence for the zxs-2 primer and zxs-3 primer are marked by asterisks; the newly created transcriptional termination signal (TTTTTAT) is shown in bold type.

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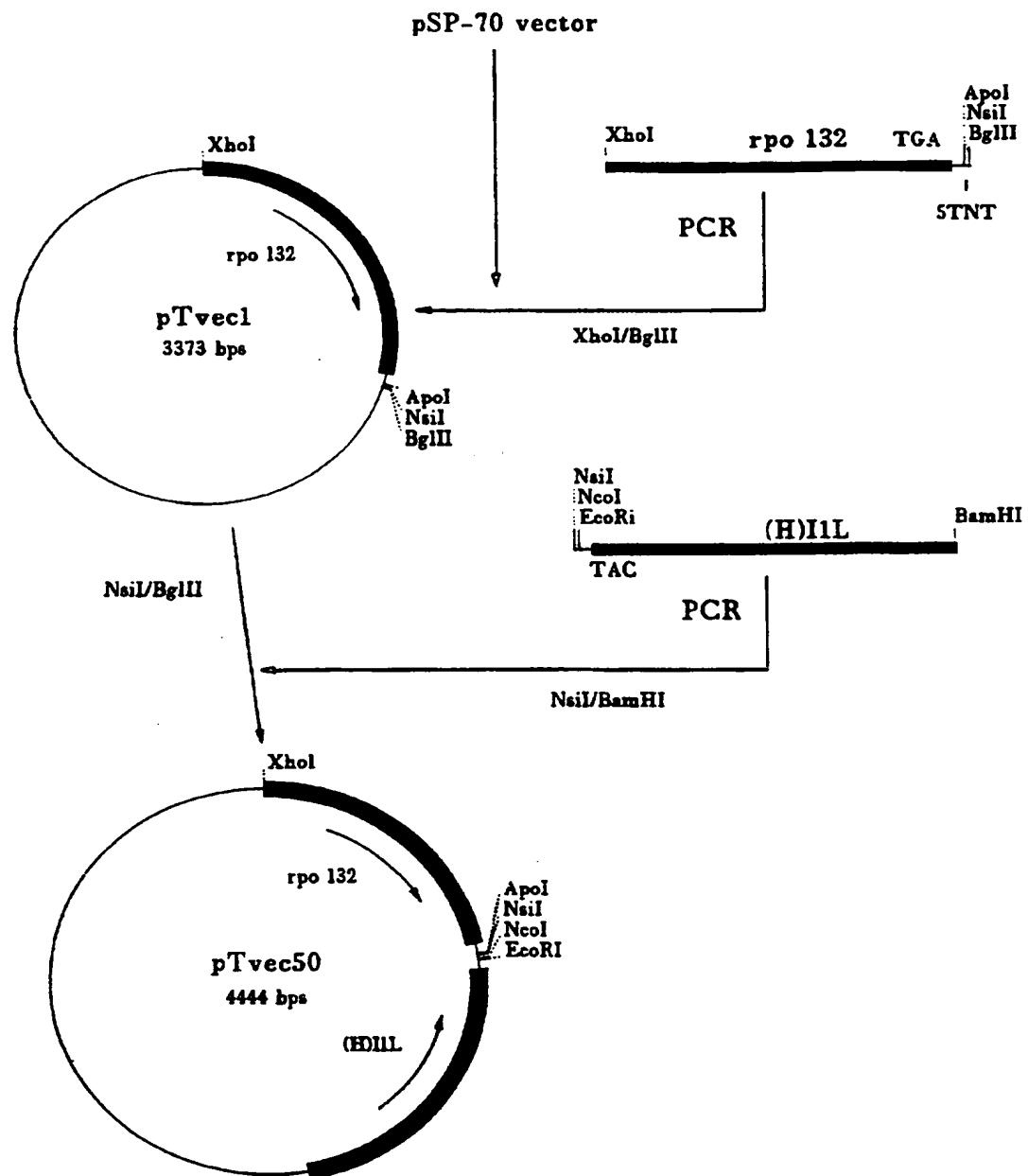
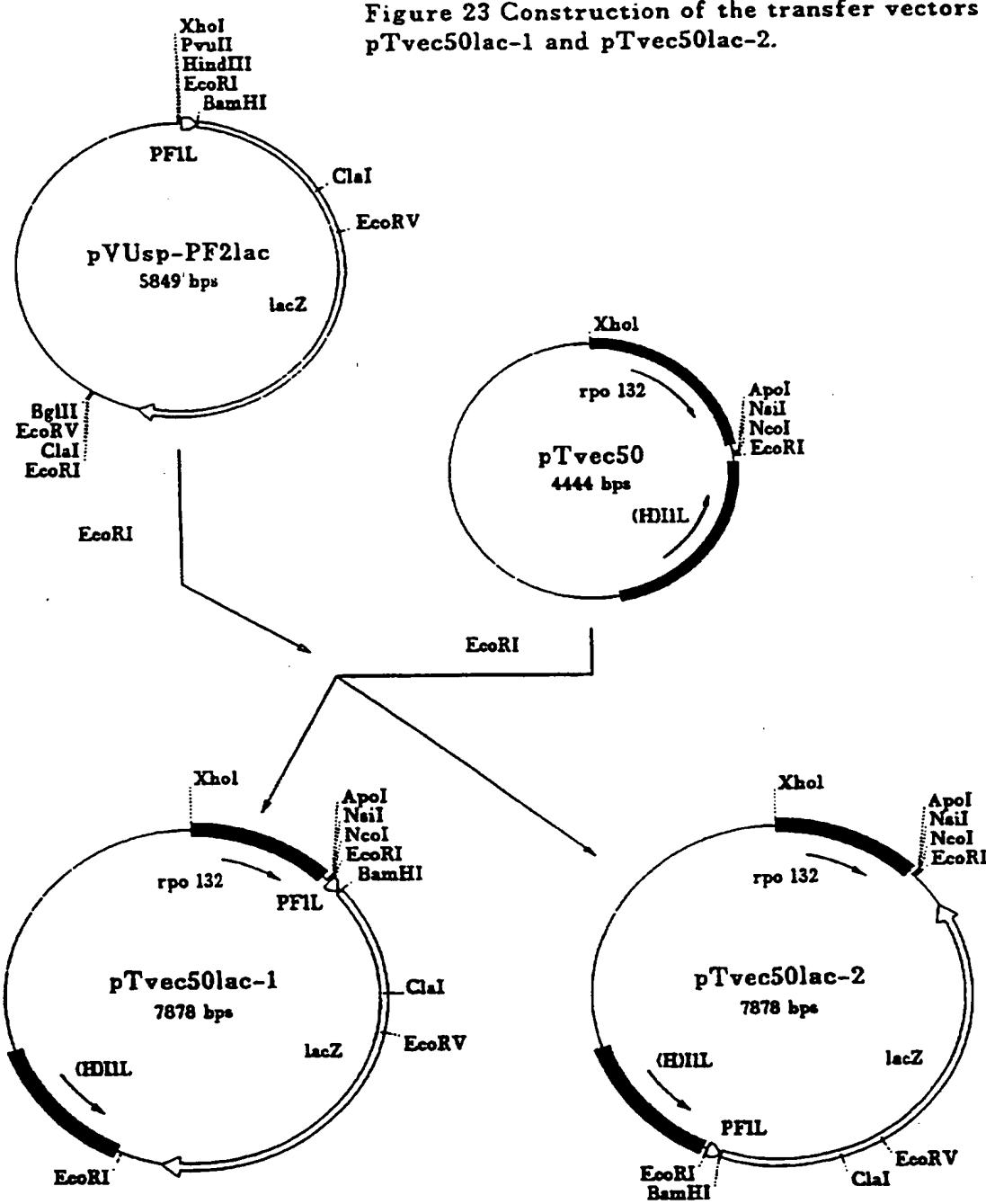


Figure 22 PCR amplification steps involved in the construction of pTvec-1 and pTvec-50.

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Figure 23 Construction of the transfer vectors
pTvec50lac-1 and pTvec50lac-2.



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/NZ 97/00040

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁶: C12N 15/86. 5/10; A61K 39/275

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE DATABASES BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT - Parapox or orfvirus: or parapoxvirus: or orf () virus: or orfv; CHEMICAL ABSTRACTS - Parapoxvirus;
MEDLINE - Parapoxvirus or orf () virus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RECOMBINANT POXVIRUSES (1992) Chapter 9 pages 306-317 "Parapoxviruses : their biology and potential as recombinant vaccines" by Robinson AJ and Lytte DJ eds M. Binns and G. Smith CRC Press. Boca Raton. In particular pages 310-316	1-25
Y	JOURNAL OF GENERAL VIROLOGY (1995) vol. 76 pages 2969-2978 by Fleming SB et al. "Genomic analysis of a transposition-deletion variant of orf virus reveals a 3.3 kbp region of non-essential DNA" See entire document.	1-25

Further documents are listed in the continuation of Box C

See patent family annex

* Special categories of cited documents:	
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"E" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search 21 May 1997	Date of mailing of the international search report 04 JUN 1997
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer J.H. CHAN Telephone No.: (06) 283 2830

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/NZ 97/00040

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VIROLOGY (1987) vol. 157 pages 13-23 by Robinson AJ et al. "Conservation and variation in orf virus genomes" See entire document, particularly last paragraph.	1-25